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14. ABSTRACT

Epidemiological and experimental evidence supports the concept that chronic inflammation promotes and enhances cancerous growth through several key mechanisms, although these processes are not well understood. Several important mechanisms by which inflammation may initiate and support malignant progression have been previously described, such as the induction of DNA damage, the promotion of angiogenesis and new vasculature, and the production of growth and survival factors. The purpose of this study is to identify a novel mechanism by which chronic inflammation may support and advance tumor progression, through the induction and expansion of immune suppressive mechanisms. We demonstrate that chronic inflammation induces tumor-associated immune-suppression, by enhancing the accumulation of a population of immature myeloid-derived suppressor cells (MDSC), which down regulate and inhibit anti-tumor immunity, allowing for the proliferation and outgrowth of transformed cells. To study the association between inflammation and immune suppression in the context of tumor progression, the 4T1 mammary carcinoma cell line engineered to secrete the pro-inflammatory cytokine interleukin 1β (4T1/IL-1β) was used to create an inflammatory tumor microenvironment. Additionally, IL-1 receptor (IL-1R)-deficient mice, which have a reduced potential for inflammation, and IL-1 receptor antagonist (IL-1Ra)-deficient mice, which have an increased potential for inflammation, were used to modulate the inflammatory milieu, and the effects of inflammation on primary and metastatic tumor progression and immune suppression were examined. The presence of IL-1β in the tumor microenvironment promotes the induction and expansion of a more potent suppressive population of MDSC, thereby enhancing tumor growth and reducing survival. Alternatively, a reduction in inflammation, in IL-1Rdeficient mice, significantly delays MDSC induction, leading to a reduction in both primary and metastatic tumor growth. Furthermore, we demonstrate that chronic inflammation promotes and enhances potent type-2 immune responses by MDSC through a mechanism dependent on the Toll-like receptor 4 (TLR4) pathway. Collectively these findings demonstrate that chronic inflammation promotes malignancy by enhancing immune suppression, through the rapid induction and expansion of a more potent population of MDSC, thus inhibiting tumor immune surveillance mechanisms, resulting in enhanced malignant progression.

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Introduction

Epidemiological and experimental observations support the hypothesis that chronic inflammation contributes to the development and progression of cancers (1-4); however the mechanisms underlying the relationship between inflammation and breast cancer are poorly understood. Inflammatory components have been shown i) to induce DNA damage which contributes to genetic instability and transformed cell proliferation (3); ii) to promote angiogenesis and thereby enhance tumor growth and invasiveness (4); and iii) to impair myelopoiesis and hematopoiesis thereby causing immune dysfunction and inhibiting immune surveillance (5, 6). Although it is generally accepted that inflammation enhances tumor progression (3, 4, 7), the mechanisms by which inflammation mediates its effects are not well understood.

Tumor progression in many patients and experimental animals with cancer is frequently associated with the expansion of a population of cells of myeloid origin, termed Myeloid-derived Suppressor Cells (MDSC). MDSC accumulate in the spleen, blood and lymphoid organs as immature myeloid cells, characterized by the surface expression of CD11b and Gr1 markers. In tumor-free individuals these immature cells differentiate into granulocytes, macrophages and/or dendritic cells (Figure 1). However, in tumor-bearing individuals, differentiation is blocked by tumor-secreted factors that impair hematopoiesis and lead to the accumulation of MDSC (5). These MDSC have potent immunosuppressive activity and inhibit both adaptive and innate immunity by preventing the activation of $CD4^+$ and $CD8^+$ T cells, reducing the number of mature dendritic cells, suppressing NK cell cytotoxicity, and by skewing immunity towards a type-2 phenotype (6, 8, 9).

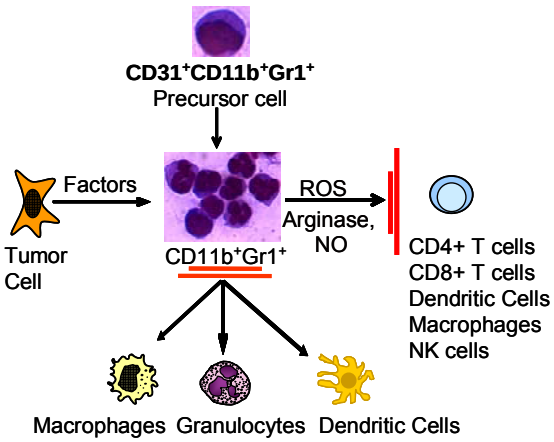

Figure1. Tumor-secreted factors promote the accumulation of myeloid-derived suppressor cells (MDSC) and inhibit myeloid cell development and differentiation.

We hypothesize that chronic inflammation causes an increase in MDSC which inhibit immune surveillance and anti-tumor immunity, thereby facilitating malignant cell transformation and proliferation. To study these mechanisms we have developed an experimental system in which tumors constitutively express the pro-inflammatory cytokine, interleukin 1β (IL-1β), resulting in an inflammatory microenvironment at the tumor site (9). We have used IL-1β because it is a key cytokine in mediating an inflammatory response (10) and has been previously shown to promote primary tumor growth (11-13) and to enhance metastatic disease (14, 15). The spontaneously metastatic

BALB/c-derived 4T1 mammary carcinoma (16-18) was used because breast cancer is one of the cancers for which inflammation is associated with poor prognosis (6), and because we want to study the effects of inflammation on the progression of both primary and metastatic tumor. Additionally, we have used knock-out mice deficient in the IL-1 receptor (IL-1R$^{-/-}$), which have a reduced inflammatory response, and mice deficient for the IL-1 receptor antagonist (IL-1Ra$^{-/-}$), which have an enhanced inflammatory response, to further modulate the inflammatory tumor microenvironment.

In addition to confirming the concept that a pro-inflammatory microenvironment enhances tumor progression and that a reduction in inflammation reduces tumor progression, our results suggest a novel mechanism by which inflammation facilitates tumor growth. We demonstrate that inflammation in the tumor microenvironment (4T1/IL-1β) promotes tumor growth and decreases survival by enhancing the accumulation of MDSC (9). MDSC induced by inflammation are phenotypically distinct and are more potent suppressors of CD8^{+} T cells (9)and push macrophage towards type-2 responses, compared to MDSC induced by 4T1 tumor alone. We also demonstrate that limiting tumor-associated inflammation leads to a delay in MDSC induction and an associated reduction in primary and metastatic disease progression, and that the effects of IL-1β are mediated by interleukin 6 (IL-6) (19). Furthermore, we identify a mechanism by which inflammation through the Toll-like receptor 4 (TLR4) and NFκB pathway on MDSC, enhances MDSC IL-10 production, which in turn inhibits macrophage IL-12 production (20)and further skews immunity towards a type-2 response.

The finding that IL-1β up-regulates MDSC accumulation in tumor-bearing mice has led us to propose the following causal relationship linking chronic inflammation with tumor progression: As tumor cells proliferate they induce an inflammatory microenvironment consisting of IL-1β and other pro-inflammatory mediators. The persistence of these mediators causes the accumulation and retention of MDSC. The MDSC, in turn, initiate and maintain an immune suppressive state which blocks immune surveillance, thereby facilitating the survival and proliferation of transformed cells. Immune dysfunction is a significant impediment to immunotherapy; therefore the goal of this project is to understand the role of inflammation in the mechanisms underlying MDSC accumulation and immune suppression, so that novel interventional strategies may be developed.

Body

Note: Text appearing in the original statement of work (SOW) is underlined.

Task 1: Phenotypic characterization of IL-1β induced MDSC.

Final Result for Task 1 – Completed (See Midterm Report 2006).

Task 2: Functional characterization of IL-1β induced MDSC and determination of target cells which are suppressed by MDSC.

Final Result for Task 2 – Completed (See Midterm Report 2006).

Task 3: Determine if IL-1β directly stimulates the accumulation of MDSC.

Final Result for Task 3 – Completed (See Midterm Report 2006).

Task 4: Test cytokines downstream if IL-1β for their ability to induce MDSC accumulation.

Final Result for Task 4 – Completed (See Midterm Report 2007).

Task 5: Determine if macrophages are involved in MDSC accumulation.

Final Result for Task 5 – Completed (See Midterm Report 2007).

Task 6: Determine if blocking inflammatory responses prevents, delays, or minimizes tumor progression.

a. Administer Celecoxib to BALB/c mice continuously, starting at time of 4T1 tumor inoculation. Monitor tumor onset, growth, metastatic load, and MDSC accumulation (24-36 months).

Results: To determine whether blocking inflammation reduces tumor growth, metastases and the accumulation of MDSC, the selective COX-2 inhibitor, SC58236, was administered to tumor-bearing mice at the time of tumor inoculation. BALB/c mice were inoculated with either 4T1 or 4T1/IL-1β tumors, and SC58236 injections were given three times per week to mice in the experimental group, while mice in the control group did not receive SC58236. Mice from both groups were tail bled once a week to measure the percent of blood MDSC as a function of tumor size, and mice were sacrificed when moribund to quantify lung metastases. Treatment of both 4T1 and 4T1/IL-1β tumor bearing mice with SC58236 decreased tumor growth and the accumulation of blood MDSC compared to control mice. The decrease in tumor progression in the experimental group was accompanied by a decrease in the number of lung metastases. The results demonstrate that treatment with a selective COX-2 inhibitor delays tumor growth, metastatic disease, and the accumulation of MDSC in both 4T1 and 4T1/IL-1β tumor-bearing mice further supporting the concept that inflammation promotes tumor progression.

Final Result for Task 6 – Completed.

Task 7: Determine if reducing inflammatory responses minimizes or delays tumor progression and immune suppression.

Final Result for Task 7 – Completed (See Midterm Report 2007).

Task 8: Determine whether hematopoietic or non-hematopoietic cells respond to tumor-derived IL-1beta.

Final Result for Task 8 – Completed (See Midterm Report 2007).

Task 9: Determine if interleukin 6 (IL-6) is a mediator of the tumor promoting effects IL-1beta.

a. Create stable 4T1 cell lines which secrete IL-6 (4T1/IL-6), by transfection with an IL-6 plasmid containing IL-6 gene (12-24 months).
b. Quantify IL-16 secretion of 4T1 stable transfectants by ELISA (12-24 months).
c. Inoculate BALB/c and IL-1R-deficient mice with 4T1/IL-6 tumor cells. Monitor tumor onset, growth, metastatic load, and MDSC accumulation (12-24 moths).

Results: To evaluate the effects of another pro-inflammatory cytokine, interleukin-6 (IL-6), as a potential candidate mediator of the effects of IL-1β on tumor progression and immune suppression. The tumor promoting and immune suppressive effects of IL-1β are not direct and involve an intermediate mediator, as neither the tumor cells nor the MDSC express the IL-1R. The secretion of IL-1β leads to the production of IL-6, and preliminary studies demonstrate that MDSC express the IL-6R, supporting the idea that IL-1β secretion from the primary tumor acts on a host intermediate cell, leading the production and secretion of IL-6, which acts directly on the MDSC. To test this hypothesis, we have engineered 4T1 tumor cells to secrete IL-6 (4T1/IL-6) at 3000 pg/ml/24hrs/$2x10^5$ cells. 4T1/IL-6 tumor cells were inoculated into BALB/c and IL-$1R^{-/-}$ mice, and tumor growth, lung metastases, and the accumulation of MDSC were measured. 4T1/IL-6 tumor cells were inoculated into BALB/c mice to evaluate the effect of another pro-inflammatory cytokine on tumor progression and immune suppression to determine whether IL-6 also enhances tumor growth and MDSC induction. Inoculation of 4T1/IL-6 tumor cells in IL-$1R^{-/-}$ mice was used to determine whether any effects due to IL-6 require IL-1 signaling, since IL-1β activation leads to the production of IL-6.

A reduction in tumor growth is observed in IL-$1R^{-/-}$ mice and may be due directly to the loss of the effects of IL-1β or may be due to the loss of a downstream product of the IL-1 signaling pathway. A potential downstream candidate is the pro-inflammatory cytokine, IL-6, whose production is reduced in IL-$1R^{-/-}$ tumor tissue **(Appendix B, Figure 1B)**. If IL-6 is a downstream mediator by which IL-1β alters MDSC activity and tumor progression, then IL-6 production should restore elevated MDSC levels and more rapid tumor progression in IL-1R-deficient mice. To test this hypothesis, IL-$1R^{-/-}$ and BALB/c mice were inoculated with 4T1/IL-6 tumor cells and tumor progression was monitored. No delay in tumor growth was observed in IL-$1R^{-/-}$ mice bearing 4T1/IL-6 tumors compared to the significant delay seen in IL-$1R^{-/-}$ mice with 4T1 tumors **(Appendix B, Figure 6A)** (19). Additionally, 4T1/IL-6 tumors in IL-$1R^{-/-}$ mice grew at a similar rate to 4T1 and 4T1/IL-6 tumors in BALB/c mice. Therefore, IL-6 compensates for the loss of IL-1R, supporting the hypothesis that IL-6 is a downstream mediator of IL-1β that facilitates tumor progression.

Since IL-6 restores primary tumor growth in IL-1R-deficient mice, we examined whether IL-6 also eliminates the reduction in lung metastases. BALB/c and IL-$1R^{-/-}$ mice were inoculated with 4T1/IL-6 tumor cells on day 0 and sacrificed on day 39-40. Lung

metastases were quantified as previously described and compared to the levels of lung metastases in BALB/c and IL-1R$^{-/-}$ mice inoculated with 4T1 parental cells **(Appendix B, Figure 6B)**. IL-6 restores the number of lung metastases in IL-1R-deficient mice to that seen in BALB/c mice, demonstrating that IL-6 may be a downstream mediator of IL-1 for metastatic dissemination to the lungs (19).

The delay in MDSC accumulation in IL-1R$^{-/-}$ tumor-bearing mice and the IL-1β-induced induction of MDSC suggest that inflammation through the production of IL-1β promotes the accumulation of MDSC and contributes to tumor progression. However, MDSC do not express the IL-1R (9)and therefore can not respond directly to IL-1β. Therefore, it is likely that IL-1β indirectly affects MDSC activity via mediators downstream of IL-1β. Because IL-6 compensates for the loss of IL-1 with respect to primary and metastatic tumor growth, we examined whether IL-6 might be a downstream mediator that affects MDSC. If IL-6 is a downstream mediator, then secretion of IL-6 by 4T1 tumor cells could compensate for the delay in MDSC accumulation in IL-1R-deficient mice. To test this possibility, BALB/c and IL-1R$^{-/-}$ mice were inoculated with 4T1 or 4T1/IL-6 tumor cells at day 0, periodically tail bled, and the percentage of blood MDSC determined by flow cytometry **(Appendix B, Figure 6C)**. On days 14-16 when the delay in MDSC in IL-1R$^{-/-}$ is most pronounced, there is no observed delay in IL-1R$^{-/-}$ mice inoculated with 4T1/IL-6 tumor cells, suggesting that IL-6 production compensates for the loss of IL-1 signaling (19).

These data demonstrate that the presence of tumor secreted IL-6 can compensate for the loss of IL-1 signaling in both primary and metastatic tumor growth and in the induction of immune suppressive MDSC. Taken together, these results implicate IL-6 in the IL-1 signaling pathway, suggesting that the indirect effects of IL-1β on tumor progression and MDSC accumulation function at least in part through IL-6. Our finding that MDSC express the IL-6R and that tumor-secreted IL-6 at least partially restores MDSC accumulation and enhanced tumor progression in IL-1R-deficient mice, confirms that IL-6 is likely to be a relevant IL-1β downstream mediator.

Final Result for Task 9 – Completed.

TASK 10: Determine if inflammation-induced MDSC enhance macrophage type-2 responses.

a. Co-culture LPS and IFNgamma-activated peritoneal macrophages from BALB/c mice with MDSC from BALB/c mice inoculated with either 4T1 or 4T1/IL-1beta mammary carcinoma cells. Quantify IL-12 and IL-10 production by ELISA (24-30 months).

Macrophages can either facilitate tumor progression or tumor rejection, depending on how they have been activated. In most tumor-bearing individuals, tumor-associated macrophages (TAMS or M2 macrophages) promote tumor progression by their elevated production of IL-10 and minimal production of IL-12 and nitric oxide (NO). In vitro activation with IL-4 and IL-13 through the alternative pathway, gives a similar profile. In contrast, macrophages activated via the classical pathway with LPS and IFNγ or macrophages from IL-4Rα-deficient mice (M1 macrophages) promote tumor rejection

through their production of high levels of IL-12 and NO, and minimal amounts of IL-10 (21, 22). One of the mechanisms by which MDSC facilitate tumor progression is through their cross-talk with macrophages, resulting in a decrease in macrophage production of IL-12 (20).

To determine if inflammation is involved in the MDSC-mediated down-regulation of IL-12, IL-12 production was measured in cultures of peritoneal macrophages from BALB/c mice activated with LPS and IFNγ in the presence of MDSC. MDSC were obtained from the blood of BALB/c mice with 4T1 mammary carcinoma, or from BALB/c mice with 4T1/IL-1β tumor cells. We have previously demonstrated that 4T1/IL-1β tumors produce a heightened pro-inflammatory tumor microenvironment (9). MDSC from 4T1-bearing mice decreased macrophage production of IL-12 by 33%, while MDSC from mice with 4T1/IL-1β tumors decreased macrophage IL-12 production by 84% **(Appendix C, Figure 1).** The IL-12 is produced by the macrophages, and not by the MDSC, since MDSC in the absence of macrophages, either with or without LPS and IFNγ, do not produce IL-12. Experiments using MDSC from IL-1R antagonist-deficient ($IL\text{-}1Ra^{-/-}$) mice, which have heightened inflammation due to their inability to down-regulate IL-1β responses, showed similar down-regulation of IL-12 (data not shown). Therefore, MDSC induced in the presence of heightened inflammation are more potent inhibitors of macrophage IL-12 production, indicating that inflammation through its action on MDSC, limits production of this type 1 cytokine in tumor-bearing individuals.

The ability of MDSC to down-regulate macrophage production of IL-12 is dependent on MDSC synthesis of IL-10 (20). Therefore, inflammation may decrease macrophage IL-12 production by increasing MDSC production of the type 2 cytokine IL-10. This hypothesis was tested by measuring IL-10 in the supernatants of peritoneal macrophages co-cultured with MDSC in the presence of LPS and/or IFNγ. MDSC were obtained from the blood of BALB/c mice with 4T1 or 4T1/IL-1β tumors **(Appendix C, Figure 2A),** or from the blood of BALB/c or $IL\text{-}1Ra^{-/-}$ mice with 4T1 mammary tumors **(Appendix C, Figure 2B)**. As previously observed (20), co-cultures of MDSC from 4T1 tumor-bearing mice with classically activated macrophages produced significant amounts of IL-10. However, MDSC from tumor-bearing mice with heightened inflammation (i.e. BALB/c mice with 4T1/IL-1β tumors, or $IL\text{-}1Ra^{-/-}$ mice with 4T1 tumors), produced significantly more IL-10. Additionally, these MDSC from heightened inflammatory environments and activated with LPS and IFNγ in the absence of macrophages produced significantly more IL-10, than MDSC from less inflammatory environments (i.e. MDSC from BALB/c mice with 4T1 tumors). Previous experiments using IL-10-deficient macrophages and MDSC demonstrated that the IL-10 in macrophage-MDSC co-cultures is produced by the MDSC, and not by the macrophages (20). This conclusion was supported by the current findings because LPS and IFNγ-activated macrophages did not express significant amounts of IL-10.

These results demonstrate that inflammation exacerbates MDSC production of IL-10, and enhances the ability of macrophages to up-regulate MDSC production of IL-10, thereby further skewing immunity towards a type 2 tumor-promoting phenotype by decreasing macrophage IL-12 production.

Final Result for Task 10 – Completed.

TASK 11: Determine if cross-talk between MDSC and macrophages is TLR4-dependent.

a. Determine the surface expression of TLR4, CD14 and CD11b on MDSC from BALB/c mice and TLR4-deficient mice inoculated with 4T1 or 4T1/IL-1beta carcinoma cells (24-30 months).
b. Quantify the expression of activated NFκB by Western Blot (24-36 months).
c. Co-culture peritoneal macrophages from BALB/c mice with MDSC from TLR4-deficient BALB/c mice inoculated with 4T1 mammary carcinoma cells. Co-culture peritoneal macrophages from TLR4-deficient BALB/c mice with MDSC from BALB/c mice inoculated with 4T1 mammary carcinoma cells. Quantify IL-12 and IL-10 production by ELISA (24-36 months).

Inflammation up-regulates CD14 expression on MDSC

LPS activation occurs through the binding of LPS to the LPS binding protein (LBP) which transfers LPS to CD14 (reviewed in (23)). CD14 associates with the TLR4 and other co-receptors to mediate LPS signaling (24-26). Signaling through this pathway activates NFκB, and leads to the induction of several inflammatory mediators, including IL-1, IL-6, COX-2, and iNOS (reviewed in (27)). The LPS/TLR4 signaling pathway is well documented in macrophages and dendritic cells; however, it has not been previously described in MDSC. To determine whether inflammation up-regulates IL-10 in MDSC through this pathway, $Gr1^+CD11b^+$ MDSC **(Appendix C, Figure 3A)** from the blood of 4T1 and 4T1/IL-1β BALB/c tumor-bearing mice, and MDSC from 4T1 $TLR4^{-/-}$ tumor-bearing mice were cultured in the presence or absence of LPS and IFNγ, and the expression of TLR4 and CD14 was determined by flow cytometry. TLR4 expression is equivalent on MDSC induced by 4T1 and 4T1/IL-1β tumor cells and does not change with LPS and IFNγ treatment (**Appendix C, Figure 3B**). In contrast, CD14 expression is elevated by LPS and IFNγ treatment, and is highest on $Gr1^+CD11b^+$ MDSC induced by 4T1/IL-1β tumor cells (**Appendix C**, **Figure 3C**). MDSC from TLR4-deficient mice, which are unresponsive to LPS (28), do not significantly up-regulate CD14. Therefore, inflammation increases MDSC expression of CD14 through a TLR4-dependent mechanism

Since CD11b is also known to interact with TLR4 (29) and to enhance responsiveness to LPS (30), MDSC induced by 4T1 or 4T1/IL-1β tumor cells in wild type BALB/c or $TLR4^{-/-}$ mice were also analyzed for expression of CD11b (**Appendix C, Figure 3D**). CD11b levels are elevated by LPS and IFNγ treatment; however, there is no difference in expression of CD11b based on the type of tumor or host, suggesting that heightened inflammation does not mediate its effects on MDSC through differential regulation of CD11b expression. Therefore, LPS plus IFNγ increase CD14 expression on MDSC, suggesting that inflammation may mediate its effects on MDSC through the CD14/TLR4 pathway.

Inflammation activates NFκB in MDSC

Signaling through the TLR4 pathway leads to the activation of NFκB which in turn activates genes which contribute to inflammation. NFκB is sequestered in the cytoplasm in association with the inhibitory subunit IκBα, but is released for translocation to the nucleus by phosphorylation and subsequent proteosomal degradation

of IκBα (reviewed in (31)). If LPS and inflammation are increasing MDSC activity by signaling through the TLR4 pathway, then MDSC activated in heightened inflammatory settings may have elevated levels of activated NFκB in the nucleus. To test this hypothesis, the levels of phosphorylated IκBα (pIκBα) and total IkBα protein were measured by western blot. MDSC from BALB/c mice with either 4T1 or 4T1/IL-1β tumors were untreated or treated for 24 hours with LPS and IFNγ, and cell lysates were immunoblotted with antibodies to pIκBα or IκBα **(Appendix C, Figure 4)**. HeLa cells stimulated with TNFα were used as a positive control for expression of total IκBα protein and pIκBα. Untreated MDSC from BALB/c mice with 4T1 or 4T1/IL-1β tumors expressed similar levels of total IκBα protein; however, MDSC from mice with 4T1/IL-1β tumors expressed 3 times more pIκBα than MDSC from mice with 4T1 tumors (ratio of IκBα to pIκBα: 0.1 and 0.299 for 4T1 and 4T1/IL-1β MDSC, respectively). Similarly, MDSC from mice with 4T1/IL-1β tumors stimulated with LPS and IFNγ expressed elevated levels of pIκBα compared to MDSC from mice with 4T1 tumors (ratio 0.37 and 0.2, respectively). MDSC induced by heightened inflammation also have elevated levels of pIκBα, and these levels are enhanced by treatment with LPS and IFNγ. Therefore, inflammation activates NFκB in MDSC, and is likely to result in the up-regulation of additional pro-inflammatory mediators.

Inflammation-induced increase of IL-10 in MDSC and decrease of IL-12 in macrophages are TLR4-dependent

If inflammation is increasing MDSC production of IL-10 through a CD14/TLR4/NFκB signaling pathway, then MDSC from TLR4-deficient tumor-bearing mice with heightened inflammation should not have elevated levels of IL-10. Likewise, if MDSC reduce macrophage production of IL-12 through a CD14/TLR4 signaling pathway, then MDSC from TLR4-deficient tumor-bearing mice should not decrease macrophage production of IL-12. This hypothesis was tested by comparing IL-10 and IL-12 levels in LPS and IFNγ-treated co-cultures of BALB/c macrophages with MDSC from BALB/c mice with 4T1 or 4T1/IL-1β tumors, or with MDSC from $TLR4^{-/-}$ mice with 4T1 tumors. As previously observed in Figure 2, LPS-activated, inflammation-induced MDSC produce elevated levels of IL-10 both in the presence and absence of macrophages (4T1/IL-1β vs. 4T1 MDSC). In contrast, MDSC from $TLR4^{-/-}$ mice produce no IL-10, regardless of the presence of LPS and IFNγ, or inflammation (**Appendix C, Figure 5A**). Similarly, inflammation-induced MDSC from BALB/c mice are very potent inhibitors of macrophage production of IL-12 (4T1/IL-1β vs. 4T1 MDSC), as previously seen in Figure 1, whereas MDSC from $TLR4^{-/-}$ mice are much less effective in reducing macrophage production of IL-12 (**Appendix C, Figure 5B**). Therefore, inflammation enhances the ability of MDSC to secrete IL-10 and to down-regulate macrophage production of IL-12 through an LPS/TLR4-dependent pathway in the MDSC.

Macrophage induction of IL-10 by MDSC is TLR4 dependent

To determine if the effects of macrophages on MDSC are also regulated through the LPS/TLR4/NFκB pathway, IL-10 and IL-12 levels were measured in co-cultures of $TLR4^{-/-}$macrophages and MDSC from BALB/c mice with 4T1 tumors (**Appendix C,**

Figure 5C). As expected, LPS-treated TLR4$^{-/-}$ macrophages do not produce significant levels of IL-12 because they are unable to respond to LPS. In contrast to earlier findings with BALB/c macrophages (**Appendix C, Figure 2**), TLR4$^{-/-}$ macrophages do not increase MDSC production of IL-10. Therefore, the cross-talk between MDSC and macrophages that promotes a tumor-promoting cytokine phenotype of high IL-10 and low IL-12 is regulated by the TLR4 signaling pathway in both MDSC and macrophages.

Key Research Accomplishments

- 4T1 cells were engineered to secrete IL-1β (4T1/IL-1β) and 4T1 cells were engineered to secrete IL-6 (4T1/IL-6) and were tested by ELISA.
- The 4T1/IL-1β MDSC were phenotyped and compared to the 4T1 MDSC phenotype: phenotypic differences were observed suggesting different subpopulations.
- The 4T1/IL-1β MDSC were functionally analyzed and compared to 4T1 MDSC: 4T1/IL-1β MDSC were more suppressive towards CD8^{+} T cells than 4T1 MDSC and suppressed through a different arginase-independent mechanism.
- ROS production by 4T1 and 4T1/IL-1β MDSC was quantified: 4T1/IL-1β MDSC produced higher levels of ROS.
- The ability of 4T1 and 4T1/IL-1β MSC to respond directly to tumor-secreted IL-1β was evaluated: neither 4T1 nor 4T1/IL-1β express the IL-1R Type I.
- Serum and tumor tissue concentrations of IL-1β were measured: no IL-1β was detected in serum of 4T1 or 4T1/IL-1β inoculated mice, while IL-1β was detected in tumor tissue of 4T1/IL-1β inoculated mice.
- Serum and tumor tissue of 4T1, 4T1/IL-1β, and IL-1R$^{-/-}$ tumor bearing mice were analyzed for a variety of inflammatory and immunosuppressive cytokines: 4T1/IL-1β tumor tissue had enhanced expression of inflammatory cytokines and IL-1R$^{-/-}$ tumor tissue had reduced expression of inflammatory cytokines.
- The expression of the IL-6 receptor was measured on MDSC: MDSC from 4T1 and 4T1/IL-1β tumor bearing mice express the IL-6 receptor.
- The effect of MDSC from 4T1 and 4T1/IL-1β tumor bearing mice on macrophages was determined: both types of MDSC reduce IL-12 production by macrophages, although 4T1/IL-1β MDSC are more potent suppressors of IL-12 production on a per cell basis.
- MDSC were demonstrated to skew immunity towards a type 2 response by the secretion of IL-10 upon LPS stimulation, and 4T1/IL-1β MDSC secreted significantly more IL-10 than 4T1 MDSC on a per cell basis.
- Tumor growth in the IL-1R$^{-/-}$ mice was evaluated: a significant delay in tumor growth was observed in IL-1R$^{-/-}$ mice with 4T1 tumors compared to BALB/c mice with 4T1 tumors.
- Tumor growth in the IL-1R$^{-/-}$ bone marrow chimeras was evaluated to determine whether host hematopoietic or non-hematopoietic cells respond to IL-1: IL-1 nonresponsiveness in either hematopoiteic of non-hematopoietic cells is sufficient to delay tumor progression.

- The development of lung metastases in the IL-1R$^{-/-}$ mice was evaluated: a significant reduction of lung metastases was observed in the IL-1R$^{-/-}$ mice with 4T1 tumors compared to BALB/c mice with 4T1 tumors.
- The development of lung metastases in the IL-1R$^{-/-}$ bone marrow chimeras was evaluated: IL-1 nonresponsiveness in both hematopoietic and non-hematopoietic cells is required for a reduction in the number of lung metastases.
- The induction of MDSC in the IL-1R$^{-/-}$ mice was measured: a significant delay in the accumulation of blood MDSC was observed in the IL-1R$^{-/-}$ mice.
- MDSC induction in the IL-1R$^{-/-}$ bone marrow chimeras was measured: non-hematopoietic and hematopoietic cells respond to IL-1 and mediate MDSC accumulation.
- The phenotypic and functional similarity between MDSC in BALB/c and IL-1R$^{-/-}$ mice with 4T1 tumors was evaluated: both populations of MDSC are phenotypically and functionally similar.
- The ability of MDSC from 4T1/IL-1β tumor bearing mice to skew macrophage responses towards type 2 responses was evaluated: 4T1/IL-1β MDSC significantly reduce macrophage production of IL-12 upon classical activation.
- Production of type 2 cytokine IL-10 by MDSC was evaluated: 4T1/IL-1β MDSC secrete enhanced IL-10 upon LPS and IFNγ treatment.
- MDSC production of IL-10 and reduction of macrophage IL-12 in IL-1ra$^{-/-}$ mice was evaluated: IL-1ra$^{-/-}$ which have an increased propensity for inflammation have MDSC which produce elevated levels of IL-10 and are more potent at reducing macrophage IL-12 production.
- The upregulation of IL-10 production by MDSC through the TLR4 pathway was evaluated: LPS upregulates the expression of CD14, the LPS co-receptor, on 4T1/IL-1β MDSC but does not upregulate CD14 on 4T1 MDSC.
- Activation of NFκB through the TLR4-CD14 pathway in MDSC was determined: 4T1/IL-1β MDSC have enhanced levels of phosphorylated IκBα compared to 4T1 MDSC.
- TLR4$^{-/-}$ mice were used to determine whether enhanced IL-10 production by MDSC was dependent on the TLR4 pathway: MDSC IL-10 production and the reduction in macrophage IL-12 is dependent on MDSC TLR4 expression.

Reportable Outcomes

The reported data has been presented as posters at the following meetings:

- Keystone Symposia Meeting on Inflammation and Cancer in February 2005 in Breckenridge, CO.

- CRI Cancer Vaccines Meeting in October 2005 in New York City, NY.

- Keystone Symposia Meeting on Inflammation and Cancer in February 2007 in Santa Fe, NM. The poster presented at the Keystone Symposia Meeting was awarded an NCI Scholar Travel Award for $1000.

- Mechanism and Therapeutic reversal of Immune Suppression in Cancer meeting in January 2007 in Clearwater, FL

The following papers were published:

- Bunt, S.K., et al. "Inflammation induces myeloid-derived suppressor cells that facilitate tumor progression". J Immunol. 2006 Jan 1;**176**(1): 284-90.

- Bunt, S.K., et al. "Reduced Inflammation in the Tumor Microenvironment Delays the Accumulation of Myeloid-derived Suppressor Cells and Limits Tumor Progression". Cancer Res. 2007; **67**: 10012-10018.

A short talk entitled "Inflammation Induces Myeloid-Derived Suppressor Cells that Facilitate Tumor Progression" was presented at the American Association of Cancer Research (AACR) Annual Meeting in April 2006 in Washington D.C.

Conclusions

The purpose of this project is to determine the mechanisms by which inflammatory factors, such as IL-1β, promote tumor-associated immune suppression in breast cancer. We propose a novel hypothesis by which inflammation promotes tumor progression by enhancing immune suppression, thereby inhibiting natural immune surveillance and allowing for the outgrowth and proliferation of transformed cells. Delineating the mechanisms by which inflammation enhances tumor-associated immune suppression and the factors that induce dysfunction will lead to the circumvention of suppression and will increase vaccine effectiveness and facilitate immunotherapy.

In this study, we have demonstrated a link between inflammation and the promotion of tumor progression and the induction of tumor-associated immune suppression. The presence of inflammation in the tumor microenvironment leads to significantly elevated levels of immune suppressive MDSC which are a distinct and more potent subpopulation of suppressor cells, demonstrating that inflammation induces immune suppression and suggesting that the induction of MDSC may serve as a link between inflammation and tumor progression. Alternatively, a reduction in tumor-associated inflammation through the elimination of the IL-1 signal (IL-1R$^{-/-}$ mice), leads to a reduction in both primary and metastatic tumor growth and this reduction is accompanied by a delay MDSC induction, demonstrating that limiting inflammation may reduce both tumor growth and the associated immune suppression. This study also highlights a mechanism by which inflammation may further subvert anti-tumor immunity through the TLR4-NFκB pathway. In this mechanism, inflammation-induced MDSC produced elevated levels of IL-10 in response to TLR4 and NFκB signaling, leading to a reduction in macrophage IL-12 production and promoting a predominant type-2 immune response. These results demonstrate a new pathway by which inflammation promotes

tumor progression through the induction and expansion of MDSC, thereby enhancing immune suppressive mechanisms and limiting anti-tumor immunity.

Inflammation is a significant impediment to the success of cancer therapies. The bulk of cancer therapies and vaccines have been tested in advanced cancer patients who are severely immune suppressed (32). Since these therapies rely on boosting the body's immune system, the "window" of successful immunotherapy is very narrow, as the success of cancer therapies relies on patients with early cancers who are immunocompetent. However, the combination of these cancer therapies with alternate therapies aimed at eliminating immune suppression may lead to successful cancer treatments. Therapies aimed at reducing inflammation during that critical "window" of time may in turn limit immune suppressive mechanisms, such as the accumulation of MDSC, and in conjunction with current and future cancer vaccines strategies, may lead to effective immunotherapies.

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APPENDIX A

Inflammation Induces Myeloid-Derived Suppressor Cells that Facilitate Tumor Progression

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Inflammation Induces Myeloid-derived Suppressor Cells that Facilitate Tumor Progression[1]

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ABSTRACT

Epidemiological and experimental observations support the hypothesis that chronic inflammation contributes to cancer development and progression; however the mechanisms underlying the relationship between inflammation and cancer are poorly understood. To study these mechanisms we have transfected the mouse 4T1 mammary carcinoma with the pro-inflammatory cytokine, IL-1β, to produce a chronic inflammatory microenvironment at the tumor site. Mice with 4T1/IL-1β tumors have a decreased survival time, and elevated levels of immature splenic $Gr1^{+}CD11b^{+}$ myeloid-derived cells. These cells, termed myeloid suppressor cells (MSC), are present in many patients with cancer and inhibit the activation of $CD4^{+}$ and $CD8^{+}$ T lymphocytes. 4T1/IL-1β-induced MSC do not express the IL-1 receptor, suggesting that the cytokine does not directly activate MSC. Neither T, B, nor NKT cells are involved in the IL-1β–induced increase of MSC because $RAG2^{-/-}$ and nude mice with 4T1/IL-1β tumors also have elevated MSC levels. MSC levels remain elevated in mice inoculated with 4T1/IL-1β even after the primary tumor is surgically removed, indicating that the IL-1β effect is long-lived. Collectively, these findings suggest that inflammation promotes malignancy via pro-inflammatory cytokines, such as IL-1β, that enhance immune suppression through the induction of MSC, thereby counteracting immune surveillance and allowing the outgrowth and proliferation of malignant cells.

INTRODUCTION

The concept that chronic inflammation promotes tumor progression was originally proposed by Virchow in the late 1800's (1). Epidemiological studies offer strong support for this concept. For example, the risk of some organ-specific cancers, particularly colorectal cancers, is significantly higher in individuals with chronic inflammation of the target organ (1-4). Experimental findings also support the observation that inflammation facilitates malignant growth. Inflammatory components have been shown i) to induce DNA damage which contributes to genetic instability and transformed cell proliferation (1); ii) to promote angiogenesis and thereby enhance tumor growth and invasiveness (4); and iii) to impair myelopoiesis and hematopoiesis thereby causing immune dysfunction and inhibiting immune surveillance (5, 6). Although it is generally accepted that inflammation enhances tumor progression (1, 4, 7), the mechanisms by which inflammation mediates its effects are not well understood.

To study these mechanisms we have developed an experimental system in which tumors constitutively express the pro-inflammatory cytokine, interleukin 1β (IL-1β), resulting in an inflammatory microenvironment at the tumor site. We have used IL-1β because it is a key cytokine in mediating an inflammatory response (8) and has been previously shown to promote primary tumor growth (8-11) and to enhance metastatic disease (11-13). The spontaneously metastatic BALB/c-derived 4T1 mammary carcinoma (14-16) was used because breast cancer is one of the cancers for which inflammation is associated with poor prognosis (6), and because we want to study the effects of inflammation on the progression of both primary and metastatic tumor.

In addition to confirming the concept that a pro-inflammatory microenvironment enhances tumor progression, our results suggest a novel mechanism by which inflammation facilitates tumor growth. We find that IL-1β-producing tumor cells are potent inducers of a population of immature $Gr1^+CD11b^+$ myeloid-derived cells called myeloid suppressor cells (MSC)[3]. Elevated levels of MSC are frequently found in many cancer patients where they cause a global and profound immune suppression (5, 6, 17-20). The IL-1β-induced MSC described here are effective suppressors of $CD4^+$ and $CD8^+$ T lymphocytes. The finding that IL-1β up-regulates MSC accumulation in tumor-bearing mice has led us to propose the following causal relationship linking chronic inflammation with tumor progression: As tumor cells proliferate they induce an inflammatory microenvironment consisting of IL-1β and other pro-inflammatory mediators. The persistence of these mediators causes the accumulation and retention of MSC. The MSC, in turn, initiate and maintain an immune suppressive state which blocks immune surveillance, thereby facilitating the survival and proliferation of transformed cells.

MATERIAL AND METHODS

Mice. BALB/c, D011.10 transgenic (21) (both from The Jackson Laboratory), $RAG2^{-/-}$ (Taconic), and Clone 4 transgenic (22, 23) mice were bred in the University of Maryland Baltimore County (UMBC) animal facility. BALB/c nude mice were obtained from NCI, Frederick. All animal procedures have been approved by the UMBC Institutional Animal Care and Use Committee.

Cell lines. The 4T1 mammary carcinoma cell line was maintained as described (24). The LBRM TG6 mouse T cell lymphoma cell line (from ATCC) was cultured in Iscove's modified Dulbecco's medium (Biosource) supplemented with 10% fetal clone 1 (FBP, Hyclone), 1% Glutamax (Invitrogen), 1% Gentamicin sulfate (Biosource), 1% Antibiotic-Antimycotic solution (Biosource) and 0.02 mM 2-mercaptoethanol (J.T. Baker). The 293T cells were maintained as described (25). Transductants were grown in the same medium as the parental cells supplemented with 400 μg/ml or 600 μg/ml of G418 (Sigma) for 4T1/RV and 4T1/IL-1β cells, respectively. 4T1/IL-1β cells cultured in vivo or grown as solid tumors in mice secreted similar amounts of IL-1β as measured by ELISA.

Reagents and antibodies. $OVA_{323-339}$ peptide and $HA_{518-526}$ peptide were synthesized in the Biopolymer Core Facility at the University of Maryland, Baltimore. Monoclonal antibodies Gr1-PE, CD11b-FITC, CD3-FITC, CD4-PE, CD8-PE, B220-PE, CD11c-PE, I-A/I-E-FITC, D^d-FITC, CD86-PE, CD80-FITC, CD40-PE, CD44-FITC, CD14-FITC, CD23-FITC, CD31-FITC, CD34-FITC, CD16/CD32-FITC, rat IgG2α-PE isotype control

and rat IgG2α-FITC isotype control were from BD Pharmingen. F4/80-FITC was from Caltag; PDL2-PE from eBioscience; CD83-FITC from Biocarta; and DEC205-FITC from Cedarlane. Rat anti-mouse Gr-1 (Clone RB6-8C5) and goat anti-rat IgG microbeads for MSC sorting were from BD Pharmingen and Miltenyi Biotech, respectively. Dichlorodihydrofluorescein diacetate (DCFDA) for ROS detection was from Molecular Probes.

Plasmids, retroviral vectors, and transductions. The pLXSN/ssIL-1β plasmid containing the signal sequence of the IL-1ra fused to the mature hIL-1β gene (26) was used to transduce 4T1 cells to create the 4T1/IL-1β cell line. The 4T1/RV cell line, a retroviral vector control, was created by transduction of 4T1 parental cells with the pLNCX2/AvrII plasmid (Clontech). 4T1/RV cells were identical to 4T1 parental cells in their phenotype and in vivo growth; hence the results for these lines are pooled and labeled as 4T1. Retroviral production and transduction were as previously described (25). Briefly, 293T cells were plated in six well plates at $1x10^6$ cells/well and transfected by the $CaCl_2$ method with the group-specific antigen, polymerase, and envelope genes, and pLXSN/ssIL-1β plasmid. After 48 hrs, viral supernatants were collected. To create 4T1/IL-1β cells, 4T1 cells were plated in six well plates at $1x10^5$ cells/well 15-18 hrs before the addition of polybrene (4 μg/ml) and viral ssIL-1β supernatants (500 μl/well). Cells were incubated at 37°C for 5-6 hrs, washed with sterile PBS, and cultured an additional 48 hrs before G418 selection was added.

Tumor inoculations, surgery, and metastasis (clonogenic) assay. Female 6-10 week old BALB/c mice were inoculated on day 0 in the mammary fat pad with $7x10^3$ tumor cells in 50 μl of PBS. Primary tumors were measured as described (14). Survival time indicates the day after tumor inoculation when mice are moribund and are sacrificed. For metastasis assays, lungs were harvested at the indicated times and metastatic disease was quantified using the clonogenic assay by plating cells in 6-thioguanine supplemented media (14).

Splenic MSC. $Gr1^+CD11b^+$ MSC were isolated from the spleens of tumor-bearing mice using magnetic bead sorting ("MACS," Miltenyi Scientific) as described (27).

T cell proliferation assays. The $CD4^+$ and $CD8^+$ T cell proliferation assays were performed as described (27). Briefly, DO11.10 or Clone 4 splenocytes were co-cultured with OVA or HA peptide, respectively, and irradiated MACS purified splenic MSC from BALB/c mice inoculated with 4T1 or 4T1/IL-1β cells. Cultures were pulsed with ^{3}H-thymidine on day 3 and cells were harvested 24 hours later. For the arginase and nitric oxide assays, the nitric oxide inhibitor, N^G-monomethyl-L-arginine (L- and D-NMMA), or the arginase inhibitor, N^w-hydroxyl-L-arginine (nor-NOHA) (Calbiochem) was added. Data are expressed as the average c.p.m. of triplicate wells.

Arginase Assay. Arginase was quantified by measuring the production of urea as described (27).

Flow cytometry. Cells were labeled for direct immunofluorescence as described (14), and analyzed using an Epics XL flow cytometer using the Expo32 ADC software (Beckman Coulter).

IL-1β fluorokine assay. The IL-1R fluorokine assay was performed according to the manufacturer's instructions (R&D Systems). Briefly, $1x10^5$ purified MSC from tumor-bearing mice were incubated with either 10 μl of rIL-1β-biotin or negative control biotinylated soybean protein for 1 hr at 4°C and then incubated with 10 μl of avidin-FITC for 30 min at 4°C in the dark. Cells were washed twice with 1XRDF1 buffer and analyzed by flow cytometry for IL-1R expression.

Reactive oxygen species (ROS). ROS production was measured by staining with DCFDA as described (27). To block ROS production, MSC were incubated with nor-NOHA at 37°C for 10 min prior to DCFDA addition. Cells were washed with sterile PBS and assayed by flow cytometry (27).

IL-1β ELISA. Supernatants from $5x10^5$ cells cultured in 3 ml of media for 24 hrs were frozen at -80°C until assayed using a hIL-1β ELISA kit according to the manufacturer's directions (Endogen). Plates were read at 420 nm on a Bio-Tek 311 microplate reader (Bio-tek; Winooski, VT) and quantified using a standard curve. Data are the mean ± SD of triplicate wells.

Statistical analyses. Student's two-tailed *t* test for unequal variance was performed for all figures using Microsoft Excel 2003. For Figure 4A, differences in tumor diameter in the inoculated mice were tested using a repeated measures profile analysis of variance (28, 29) using the procedure in SAS V 9.1 (SAS Institute Inc.).

RESULTS

Transduced 4T1 cells secrete mature functional IL-1β

To examine the role of inflammation and IL-1β on the development and progression of breast cancer, 4T1 mammary carcinoma cells were transduced with a construct containing the mature form of human IL-1β fused to the signal sequence from the IL-1ra (ssIL-1β construct; 4T1/IL-1β cells), allowing active hIL-1β to be secreted extracellularly (26). 4T1/IL-1β cells secreted 1000 pg/ml/$5x10^5$/24hrs as quantified by ELISA. As a control for transduction with a retroviral vector, 4T1 cells were transduced with an empty vector (4T1/RV). To evaluate whether the transduction or the secretion of IL-1β altered growth rate, 4T1 and 4T1/IL-1β cells were plated at equivalent densities and counted every 24 hours for 3 days. Both lines had the same growth kinetics, indicating that IL-1β transduction did not affect the in vitro growth rate. To further characterize the 4T1/IL-1β cells, MHC class I and class II surface expression was analyzed by flow cytometry. Transduction of 4T1 cells with ssIL-1β did not alter the amount of $H\text{-}2D^d$, and neither 4T1 nor 4T1/IL-1β cells express MHC II. MHC I and II phenotype and in vivo growth of 4T1 and 4T1/RV cells were identical, so results of these controls were pooled and are labeled as 4T1 in the following experiments.

IL-1β enhances primary tumor growth and decreases survival time

To examine the role of IL-1β on primary tumor onset and growth, 4T1 and 4T1/IL-1β cells were injected into BALB/c mice on day 0. For both groups, primary tumors were measured every 3-5 days and were palpable by day 10. 4T1/IL-1β primary tumors

grew significantly faster than parental 4T1 primary tumors ($p<0.01$). To determine whether IL-1β affects survival, 4T1 and 4T1/IL-1β injected mice were followed until they were moribund. 4T1/IL-1β injected mice had significantly reduced survival time ($p<0.01$; 26-44 days with a mean of 37 days; n=13) compared to 4T1 injected mice (33-60 days with a mean of 46 days; n=15). These data indicate that IL-1β increases the primary tumor growth rate and suggest that the difference in survival may be due to enhanced tumor progression.

IL-1β leads to an earlier and faster accumulation of myeloid suppressor cells

Because MSC frequently accumulate in tumor-bearing individuals and can reduce survival time, we examined MSC levels in mice with 4T1/IL-1β vs. 4T1 tumors. BALB/c mice were inoculated with 4T1 or 4T1/IL-1β cells and the percentage of splenic MSC was measured as a function of time after tumor inoculation. Mice with 4T1/IL-1β tumors have higher levels of MSC than mice with 4T1 tumors (Fig. 1A). Because MSC accumulation is known to increase with increasing tumor burden, mice with 4T1/IL-1β tumors may have more MSC because their primary tumors are larger. To address this question, the percentage of splenic MSC was plotted relative to tumor diameter. As seen in Fig. 1B, MSC levels are consistently higher in 4T1/IL-1β vs. 4T1 mice regardless of the size of their primary tumors. Additionally, increased levels of MSC in other organs, such as the lungs, were also elevated in 4T1/IL-1β tumor-bearing mice compared to 4T1 tumor-bearing mice (data not shown), suggesting that IL-1β secretion by the primary tumor systemically enhances MSC accumulation. These data indicate that IL-1β induces the accumulation of elevated levels of MSC.

Mice with IL-1β tumors do not have more lung metastases

To determine whether IL-1β affects the progression of lung metastases, BALB/c mice were injected with 4T1 or 4T1/IL-1β cells and lungs were harvested 5 weeks later or when the mice were moribund. The number of lung metastases was quantified using the clonogenic assay (14). Both 4T1 and 4T1/IL-1β injected mice developed lung metastases (Fig. 2). There is no difference in the number of lung metastases between 4T1 and 4T1/IL-1β injected mice at week 5; however, when the mice become moribund, 4T1/IL-1β injected mice have fewer lung metastases ($p<0.05$) compared to 4T1 injected mice. These results indicate that IL-1β does not increase the number of metastatic cells in the lungs, and suggest that IL-1β is not reducing survival time by increasing metastatic disease.

4T1 and 4T1/IL-1β cells do not express the IL-1 receptor type I

IL-1β may be mediating its effect by acting directly on 4T1 tumor cells or on host cells. To determine whether 4T1/IL-1β cells had the potential to respond to IL-1β, 4T1 and 4T1/IL-1β cells were labeled with fluorescent IL-1β protein and analyzed by flow cytometry. LBRM TG6 cells, which express the IL-1R type I, were used as positive control cells. Neither 4T1 nor 4T1/IL-1β cells express detectable IL-1R type I indicating that these cells cannot respond to IL-1β (Fig. 3). These results implicate host cells as the responders to the tumor-secreted IL-1β.

IL-1β-induced tumor growth is not dependent on host T, B, or NKT cells

Immunodeficient and knockout mice were used to determine if host immune cells are required for the IL-1β effect. Wild type BALB/c and $RAG2^{-/-}$ BALB/c mice, which are deficient for T, B, and NKT cells, were inoculated with 4T1 or 4T1/IL-1β cells and primary tumor growth was measured every 3-5 days (Fig. 4A). 4T1/IL-1β primary tumors grew significantly more rapidly in both BALB/c and $RAG2^{-/-}$ mice ($p<0.01$) and mice with 4T1/IL-1β tumors became moribund sooner than mice with 4T1 tumors. Therefore the increased tumorigenicity of 4T1/IL-1β tumor cells is not dependent on host lymphoid cells.

To determine if lymphoid cells influence metastatic disease, wild type BALB/c and $RAG2^{-/-}$ mice were inoculated with 4T1 and 4T1/IL-1β cells on day 0 and their lungs were harvested between days 25 and 35. Metastases were quantified using the clonogenic assay. IL-1β expression did not affect the average number of lung metastases in BALB/c mice, however IL-1β induced a significant increase ($p=0.05$) in the number of lung metastases in $RAG2^{-/-}$ mice (Fig. 4B). $RAG2^{-/-}$ mice with 4T1/IL-1β tumors have statistically higher number of metastases compared to BALB/c mice with 4T1/IL-1β tumors. Therefore, the presence of B, T, and NKT cells naturally limits the increase in lung metastases by IL-1β.

Enhanced splenic MSC accumulation by IL-1β does not require T, B, or NKT cells

To determine if host lymphoid cells are required for the IL-1β-induced increase in MSC, BALB/c, T cell-deficient nude, and $RAG2^{-/-}$ mice were inoculated with 4T1 or 4T1/IL-1β cells and their splenocytes harvested on day 25-35 and assayed by flow cytometry for $Gr1^{+}CD11b^{+}$ cells. All strains with 4T1/IL-1β tumors had significantly more splenic

MSC than mice with 4T1 tumors (including RAG2$^{-/-}$ with 4T1 vs. RAG2$^{-/-}$ with 4T1/IL-1β) ($p<0.01$) (Fig. 4C), confirming the findings of figure 1A and demonstrating that the ability of IL-1β to elevate MSC does not require lymphoid cells. Interestingly, BALB/c and nude mice with 4T1/IL-1β tumors had similar levels of MSC levels, while RAG2$^{-/-}$ mice with 4T1/IL-1β tumors had significantly higher numbers of MSC. Therefore, although lymphoid cells are not required for the IL-1β-induced effect, deletion of γδ T cells and/or B cells appears to facilitate an even larger accumulation of MSC.

IL-1β induced MSC are phenotypically distinct from 4T1 MSC

IL-1β may be augmenting an existing population of MSC, or it may be inducing a novel population. To distinguish between these two possibilities, BALB/c mice were injected on day 0 with 4T1 or 4T1/IL-1β cells, and splenic MSC were MACS sorted for Gr1$^+$ cells on day 35. Purified cells were >90% Gr1$^+$CD11b$^+$ as measured by flow cytometry (Fig.5A). Fig. 5B shows the phenotype of the purified Gr1$^+$CD11b$^+$ cells for various cell surface markers that have been used to characterize MSC. 4T1/IL-1β MSC expressed higher levels of CD8, CD80, CD83, and CD14 and lower levels of CD44 and B220 relative to 4T1 MSC. To evaluate whether these differences were a result of direct action of IL-1β the MSC were stained for the IL-1 Type I receptor (IL-1RI). Both 4T1 and 4T1/IL-1β-induced MSC were negative for the IL-1RI indicating that MSC cannot respond directly to IL-1β. Therefore, 4T1 and 4T1/IL-1β MSC share some common markers; however, they also have phenotypic differences.

IL-1β–induced MSC suppress CD4$^+$ and CD8$^+$ T cells

Previous data demonstrate that 4T1-induced MSC suppress both $CD4^+$ and $CD8^+$ T cells via an arginase-dependent mechanism (27). Because IL-1β–induced MSC phenotypically differ from 4T1-induced MSC, it is possible that the 4T1/IL-1β MSC are also functionally distinct. To determine if 4T1/IL-1β MSC have different functional activity from 4T1 MSC, BALB/c mice were injected with 4T1 and 4T1/IL-1β cells on day 0 and splenic MSC harvested and purified by MACS sorting on day 35, and tested for their ability to inhibit T cell proliferation. To test for suppressive activity against $CD4^+$ T cells, I-A^d-restricted, $OVA_{323-339}$ peptide-specific DO11.10 transgenic splenocytes were stimulated with $OVA_{323-339}$ peptide in the presence or absence of purified MSC from 4T1 or 4T1/IL-1β inoculated mice (Fig. 6A). Both 4T1 and 4T1/IL-1β MSC suppressed D011.10 proliferation by >95% as measured by ^{3}H-thymidine uptake. To identify the molecule mediating this suppression, L-NMMA or nor-NOHA, inhibitors of nitric oxide and arginase, respectively, were added to the D011.10 cultures. Nor-NOHA treatment partially reversed suppression by both 4T1 and 4T1/IL-1β MSC, whereas L-NMMA treatment had no effect. Arginase levels in 4T1 and 4T1/IL-1β-induced MSC were equivalent as measured enzymatically (data not shown). Therefore, 4T1/IL-1β and 4T1-induced MSC are equally suppressive for $CD4^+$ T cells, and they both at least partially mediate their effect via arginase.

To determine if IL-1β MSC also block $CD8^+$ T cell activation, $HA_{518-526}$ peptide-specific, K^d-restricted Clone 4 transgenic splenocytes were stimulated with $HA_{518-526}$ peptide and co-cultured in the presence or absence of purified 4T1 or 4T1/IL-1β MSC. Although both 4T1 and 4T1/IL-1β MSC suppress $CD8^+$ T cell activation, 4T1/IL-1β MSC are more suppressive on a per cell basis than 4T1 MSC (Fig. 6B; $p<0.01$).

However, in contrast to results of Fig. 6A, addition of the arginase inhibitor nor-NOHA does not reverse the suppression by 4T1/IL-1β MSC. Likewise, no reversal of suppression was observed when the nitric oxide inhibitor L-NMMA or its inactive enantiomer D-NMMA was added. Therefore, 4T1/IL-1β MSC are more effective suppressors of $CD8^+$ T cells than 4T1 MSC and they suppress by an arginase-independent mechanism.

IL-1β–induced MSC express higher levels of reactive oxygen species (ROS)

To determine if 4T1 and 4T1/IL-1β MSC differ in other potential effector mechanisms, ROS expression was analyzed in the two populations. MACS purified MSC from 4T1 and 4T1/IL-1β injected BALB/c mice were stained with DCFDA and analyzed by flow cytometry. Both 4T1 and 4T1/IL-1β MSC have high levels of ROS; however 4T1/IL-1β MSC contain more ROS (Fig. 6C). The addition of nor-NOHA differentially decreases, but does not eliminate ROS levels. Therefore, 4T1/IL-1β MSC express more ROS on a per cell basis than 4T1 MSC, and a significant amount of this ROS production is affected by arginase production.

4T1/IL-1β MSC remain elevated even when primary tumor is removed

Surgical excision of primary 4T1 tumor from BALB/c mice leads to a partial regression of splenic MSC (27). To determine if IL-1β affects this regression, BALB/c mice were inoculated with 4T1 or 4T1/IL-1β cells on day 0, and primary tumors were either left in place (non-surgery group) or removed (surgery group) on day 25. Both groups were sacrificed on day 35, and the percentages of $Gr1^+CD11b^+$ splenic MSC were compared.

As observed previously (27), surgical excision of the 4T1 tumor caused a significant decrease in MSC (Fig.7A; $p<0.01$). In contrast, there was no significant decrease in MSC in mice with resected 4T1/IL-1β tumors. These results imply that secretion of IL-1β by the primary tumor either irreversibly enhances MSC accumulation, or the presence of residual IL-1β prevents the regression of MSC.

To evaluate the effect of IL-1β on metastatic disease after primary tumor removal, the number of metastatic cells in the lungs of the mice in figure 7A was quantified using the clonogenic assay. No differences in lung metastases were seen (Fig. 7B), in agreement with the results of Fig. 2 that IL-1β does not impact metastatic disease.

DISCUSSION

Although the concept that chronic inflammation in the tumor microenvironment leads to increased malignant growth and tumor progression is broadly accepted, the mechanisms behind this interaction have remained elusive. The results presented here suggest that the induction of MSC by pro-inflammatory cytokines directly contributes to tumor progression by inhibiting tumor immunity. Cell-mediated immunity is known to protect against tumor onset and progression (30, 31), and MSC are known to block this process. Therefore, it is likely that the MSC induced during an inflammatory response facilitate tumor growth by inhibiting the activation and/or function of tumor-specific lymphocytes. This novel mechanism is consistent with the findings that MSC are found in many cancer patients, particularly patients with head and neck cancers, lung carcinomas, and breast cancers (5, 6, 17-19), and that chronic inflammation in some of these malignancies, such as oral, esophageal and lung cancers, may predispose to or enhance malignant growth (4). Chronic inflammation associated with infectious agents, such as *Schistosomiasis* and *Helicobacter pylori*, is also thought to predispose to malignancy (1, 3, 32, 33), and some of these agents are associated with MSC accumulation (6). Collectively, these published observations plus the results reported here, support the hypothesis that chronic inflammation facilitates tumor growth by inducing MSC that down-regulate immune surveillance, thereby providing an environment favorable for tumor progression.

Previous studies have examined the effects of IL-1β on tumorigenicity by transfecting/transducing tumor cells with this cytokine. Most investigators find that IL-1β enhances tumor progression (9, 10), although a minority of reports indicate that it

inhibits tumor growth (34, 35). These contradictory results are likely due to the different tumor systems studied and/or to the different amounts of IL-1β secreted by the various transfectants, as suggested by (8). IL-1β acts on many cell types, inducing a variety of effects including increased production of prostaglandin E2, vascular endothelial growth factor, interleukin 6, and circulating levels of granulocyte-macrophage colony-stimulating factor (36). Since all of these molecules have been suggested as inducers of MSC (5, 6), one or more of them may be the vehicle for IL-1β–induced immune suppression.

IL-1β was selected for these experiments because it is a pleiotropic cytokine that produces its effect through a wide array of mechanisms, cell types, and molecules (36). Since neither MSC nor 4T1 tumor cells contain a receptor for IL-1β, it is improbable that IL-1β is the direct inducer of MSC. More likely, IL-1β regulates MSC levels by activating a signal transduction pathway that stimulates cells which then secrete factors that directly induce MSC accumulation. Many cell types express the IL-1R type I allowing them to respond to the cytokine (8, 36), and hence are potential candidates. Alternatively, IL-1β may regulate MSC accumulation by down-regulating cells and/or factors that normally minimize MSC expansion. This possibility is supported by the findings that tumor-bearing RAG2$^{-/-}$ mice have higher levels of MSC than wild type BALB/c or nude mice, implying that γδ T cells and/or B cells normally limit the tumor-associated expansion of splenic MSC.

Although MSC are present in many tumor-bearing patients and experimental animals, there is significant phenotypic variation between MSC from different individuals (19, 27, 37, 38), suggesting there are distinct subpopulations of MSC (27).

The observation that 4T1/IL-1β-induced MSC differentially express some cell surface markers relative to 4T1-induced MSC further supports the concept of MSC heterogeneity. However, this finding also raises the question of whether over-expression of IL-1β induces a distinct MSC subpopulation, or whether it expands and modifies the MSC induced by wild type 4T1 tumor. Compared to 4T1 MSC, 4T1/IL-1β MSC expressed higher levels of CD8, CD80, and CD83, a marker expressed by mature dendritic cells and activated lymphocytes. Additionally, expression of the lipopolysaccharide receptor, CD14, usually expressed by macrophages and/or monocytes, was increased on 4T1/IL-1β MSC. 4T1/IL-1β MSC also expressed lower levels of the B cell marker, B220, and the cell adhesion marker, CD44. Although, the 4T1/IL-1β MSC phenotype suggests a partially activated cell, these surface markers do not classify the MSC into any known cellular category. It is interesting that CD14 is elevated only on 4T1/IL-1β MSC suggesting that IL-1β production stimulates CD14 expression. CD14 has been shown to couple with the Toll-like receptor 4, which can also complex with IL-1R, and leads to the activation of NFκB, cyclooxygenase-2, and prostaglandin E2 (39, 40). This pathway may be one of the mechanisms responsible for IL-1βinduced MSC accumulation and survival, and deserves further study.

Although these phenotypic differences exist, it is unclear if they are biologically significant, since 4T1 and 4T1/IL-1β-induced MSC are both highly immunosuppressive for $CD4^+$ and $CD8^+$ T cells. Experiments with wild type 4T1 tumor have shown that surgical removal of primary tumor reduces MSC levels (27) and restores host immunocompetence (41). In contrast, mice with resected 4T1/IL-1β tumors retain very

high levels of MSC, suggesting that inflammation-induced MSC are likely to be a significant obstacle to effective immunotherapy.

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[3]Abbreviations used in this study:

4T1/IL-1β,	4T1 cells transduced with ssIL-1β
4T1/RV,	4T1 cells transduced with retroviral vector
DCFDA,	dichlorodihydrofluorescein diacetate
D-NMMA,	N^G-monomethyl-L-arginine enantiomer
HA,	influenza hemagglutinin
IL-1ra,	interleukin 1 receptor antagonist
IL-1RI,	interleukin 1 receptor type I
L-NMMA,	N^G-monomethyl-L-arginine
MSC,	myeloid suppressor cell(s)
Nor-NOHA,	N^W-hydroxyl-nor-L-arginine
ROS,	reactive oxygen species
ssIL-1β,	construct expressing hIL-1β

FIGURE LEGENDS

Fig. 1: IL-1β increases the accumulation of splenic MSC. BALB/c mice were inoculated with 7000 4T1 or 4T1/IL-1β tumor cells in the abdominal mammary gland on day 0. Spleens were harvested at the indicated time points (A) or tumor diameters (B) and $Gr1^{+}CD11b^{+}$ MSC were quantified by flow cytometry. Values are the averages ± SD of 5-18 and 5-19 mice per group for (A) and (B), respectively. Data are pooled from 5 experiments for both (A) and (B). * indicates statistical significance at $p<0.01$.

Fig. 2: IL-1β does not alter the number of lung metastases. BALB/c mice were inoculated with 4T1 or 4T1/IL-1β tumor cells as in Fig. 1 and their lungs harvested at the indicated times. Metastatic cells were quantified using the clonogenic assay. Each dot represents an individual mouse. Horizontal bars indicate the mean number of metastases. 4T1 and 4T1/IL-1β groups are statistically significantly different from each other at $p<0.05$ for all time periods and tumor diameters. Data are pooled from 4 independent experiments.

Fig. 3: 4T1 and 4T1/IL-1β tumor cells do not express the IL-1 receptor type I. 4T1 and 4T1/IL-1β tumor cells were stained with avidin-FITC and either biotinylated rIL-1β protein or biotinylated soybean protein and analyzed by flow cytometry. Soybean protein expression levels were identical on 4T1 and 4T1/IL-1β tumor cells and are shown as a single peak. Data are from one of three independent experiments.

Fig. 4: T, B and NKT cells are not required for the IL-1β-induced accumulation of MSC. BALB/c and RAG2$^{-/-}$ mice were inoculated with 4T1 or 4T1/IL-1β tumor cells and monitored for primary tumor growth and survival. (A) Primary tumor diameter plotted as a function of time after tumor inoculation. Each curve represents an average of 3-8 mice. Growth of primary 4T1/IL-1β tumors is more rapid than growth of primary 4T1 tumors in both strains ($p < 0.01$). (B) Lung metastases were quantified from the mice in panel A using the clonogenic assay. Each dot represents an individual mouse. Horizontal bars indicate the mean number of lung metastasis. (C) BALB/c, nude and RAG2$^{-/-}$ mice bearing 4T1 or 4T1/IL-1β tumors were euthanized at week 5 and the percentage of splenic Gr1^{+}CD11b^{+} MSC was quantified by flow cytometry. All columns are statistically significantly different from other columns in the same strain and other strains (including RAG2$^{-/-}$ with 4T1 vs. RAG2$^{-/-}$ with 4T1/IL-1β) at $p<0.01$ except BALB/c with 4T1 vs. nude with 4T1 and BALB/c with 4T1/IL-1β vs. nude with 4T1/IL-1β.

Fig. 5: MSC from mice with 4T1/IL-1β tumors and MSC from mice with 4T1 tumors have phenotypic differences. BALB/c mice were inoculated with 4T1 or 4T1/IL-1β tumor cells and spleens were harvested when tumor diameters reached 8-10 mm. (A) MACS purified MSC were analyzed by flow cytometry for Gr1 and CD11b expression. (B) MACS purified MSC were phenotyped with mAbs to the indicated markers. Dotted peaks are controls (fluorescently labeled soybean protein for IL-1R panel or anti-rat isotype-FITC and anti-rat isotype-PE antibodies for all other panels; 4T1 and 4T1/IL-1β MSC had the same fluorescent staining for controls, hence only one peak is shown);

black solid lines and gray peaks are MSC from 4T1 and 4T1/IL-1β inoculated mice, respectively. Data are from one of three independent experiments.

Fig. 6: MSC from mice with 4T1/IL-1β tumors express increased levels of ROS and suppress $CD4^+$ and $CD8^+$ T cells. $CD4^+$ DO11.10 (A) or $CD8^+$ Clone 4 (B) transgenic splenocytes were stimulated with $OVA_{323-339}$ or $HA_{518-526}$ peptide, respectively, and co-cultured in the presence or absence of MACS-purified MSC from mice with 4T1 or 4T1/ IL-1β tumors. Inhibitors of arginase (nor-NOHA), nitric oxide (L-NMMA), or the inactive enantiomer (D-NMMA) were added to some wells. T cell proliferation was measured as c.p.m. of ^{3}H-thymidine. Data are from one of four independent experiments. In panels A and B nor-NOHA-treated groups are statistically significantly different from their untreated counterparts at $p<0.05$. In panel B, nor- NOHA-treated 4T1 MSC are also statistically significantly different from nor-NOHA-treated 4T1/IL-1β MSC at $p<0.05$ (C) MACS purified MSC from mice with 4T1 or 4T1/IL-1β tumors were incubated with DCFDA in the presence or absence of the arginase inhibitor nor-NOHA and ROS production was measured by flow cytometry. Data are from one of three independent experiments.

Fig. 7: MSC do not regress after surgical resection of primary 4T1/IL-1β tumors. BALB/c mice were inoculated with 4T1 or 4T1/IL-1β tumor cells as in Fig. 1 and the primary tumors were either left intact (non-surgery groups) or surgically excised on day 25 (surgery groups). Lungs and spleens were harvested on day 35. (A) The percent of $Gr1^+CD11b^+$ splenic MSCs was quantified by flow cytometry. Values are the averages

of 7-19 animals per group and are pooled from two independent experiments. (B) Lung metastases were quantified using the clonogenic assay. Each dot represents an individual mouse.

Figure 1: IL-1β increases the accumulation of splenic MSC

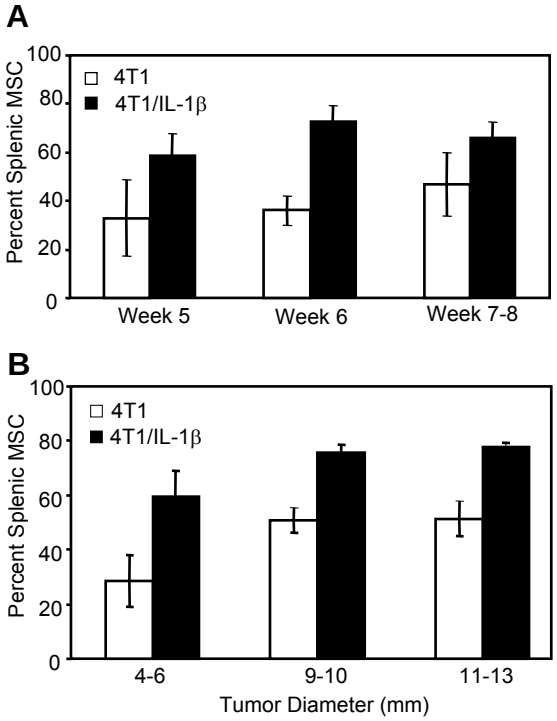


Figure 2: IL-1β does not alter the number of lung metastases

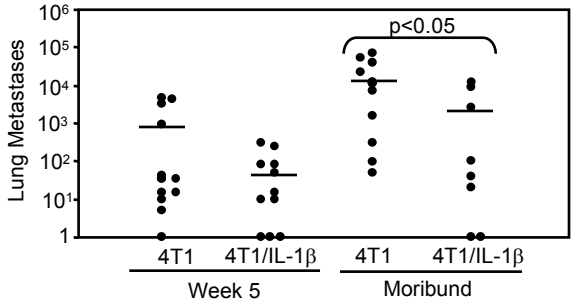


Figure 3: 4T1 and 4T1/IL-1β tumor cells do not express the IL-1 receptor type I.

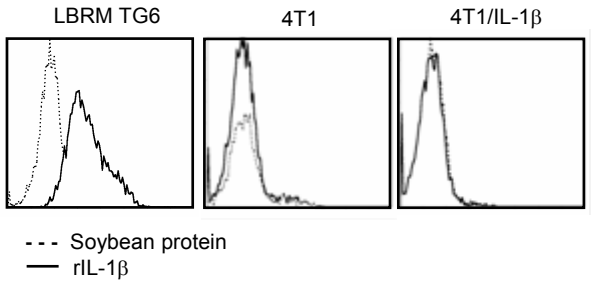


Figure 4: T, B, and NKT cells are not required for the IL-1β-induced accumulation of MSC.

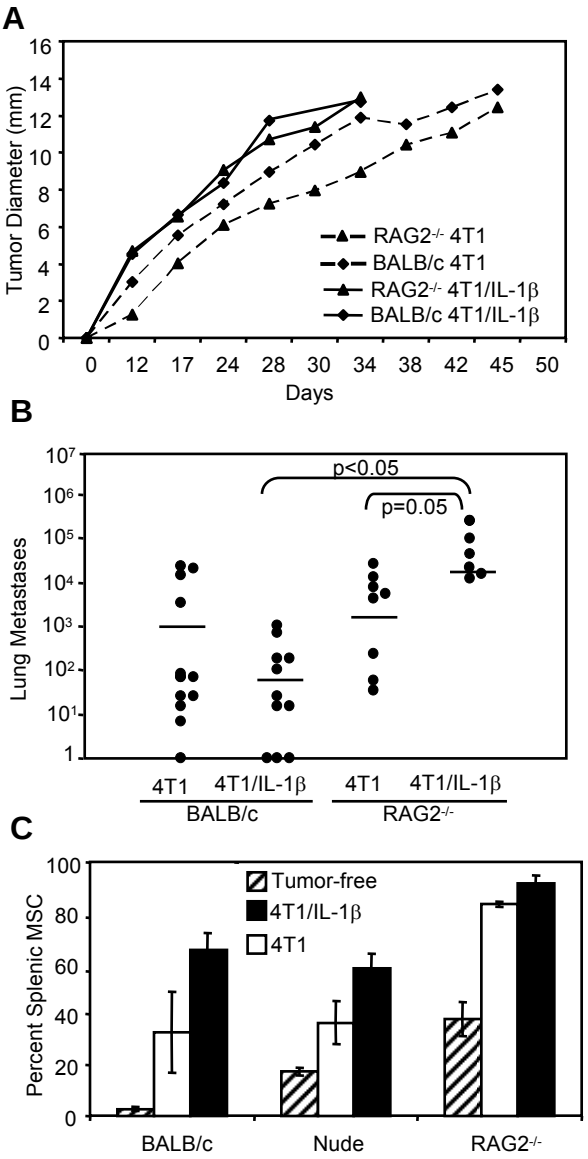


Figure 5: MSC from mice with 4T1/IL-1β tumors and MSC from mice with 4T1 tumors have phenotypic differences.

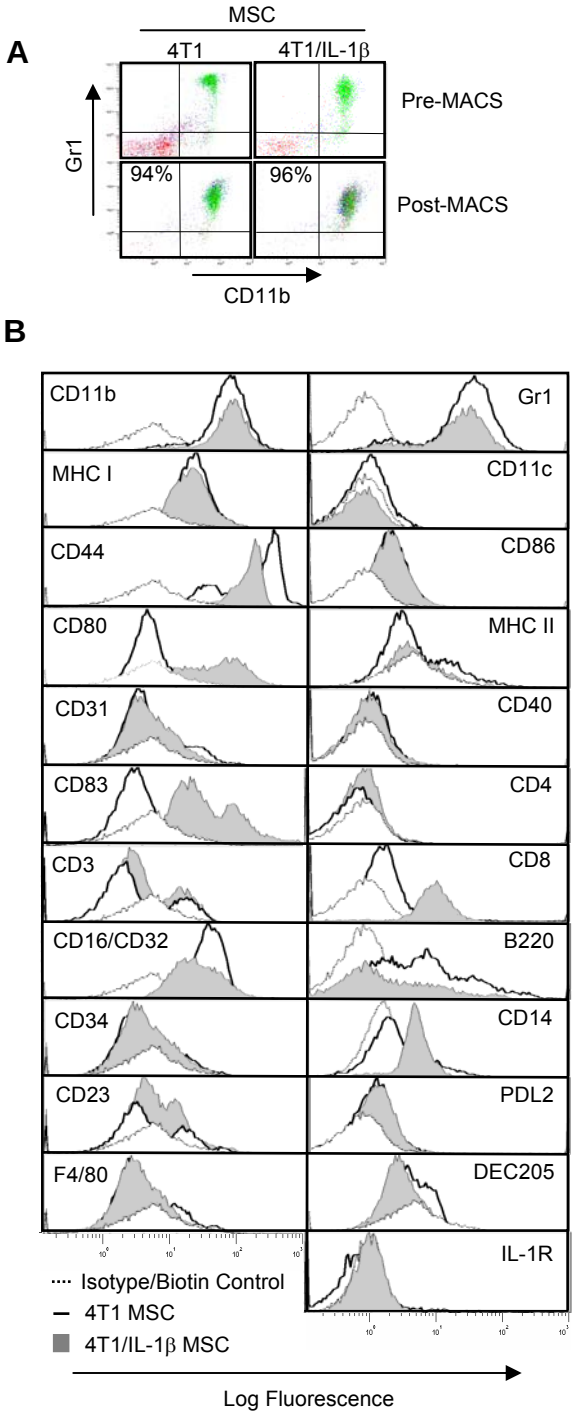


Figure 6: MSC from mice with 4T1/IL-1β tumors express increased levels of ROS and suppress $CD4^+$ and $CD8^+$ T cells.

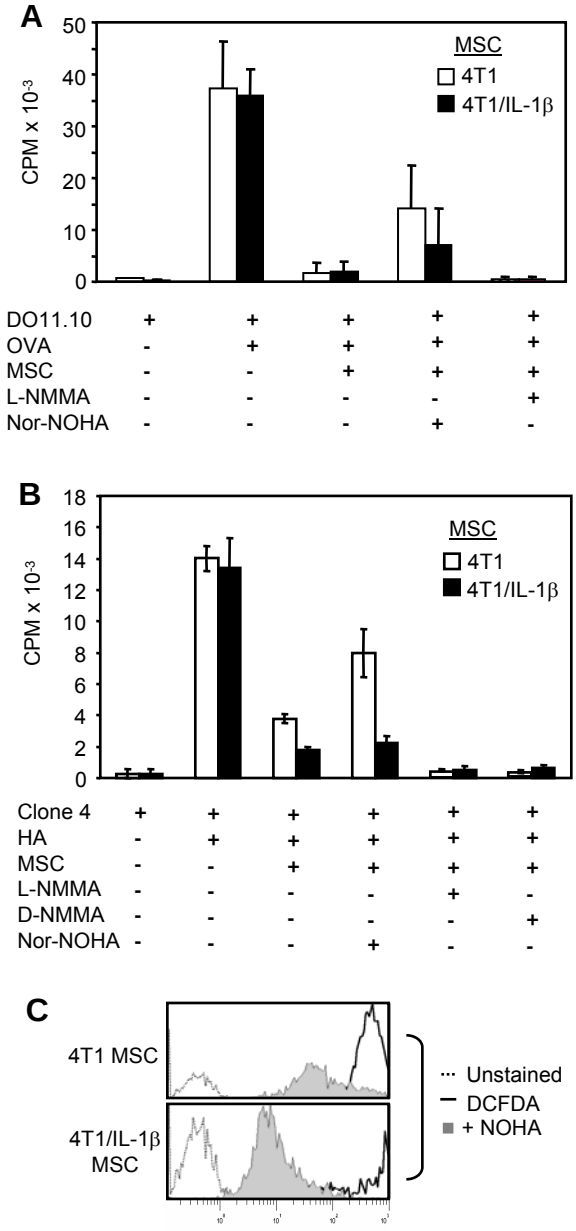
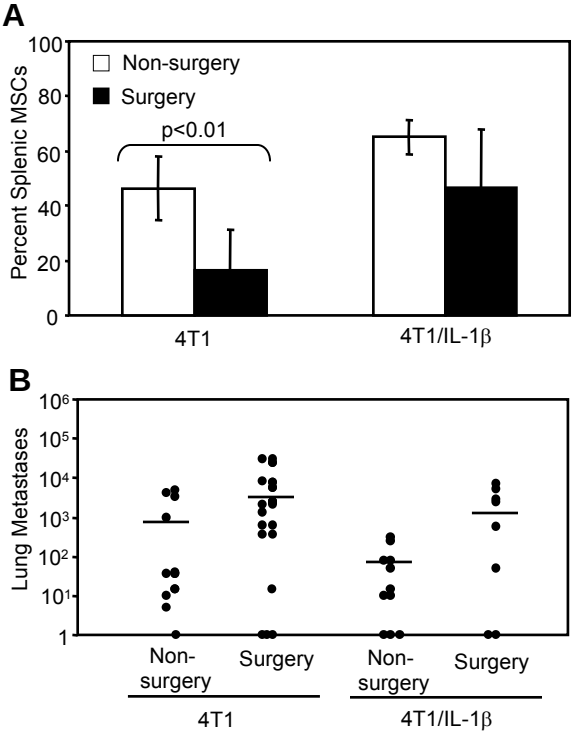


Figure 7: MSC do not regress after surgical resection of primary 4T1/IL-1β tumors.



APPENDIX B

Reduced Inflammation in the Tumor Microenvironment Delays

the Accumulation of Myeloid-derived Suppressor Cells and

Limits Tumor Progression

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Reduced Inflammation in the Tumor Microenvironment Delays the Accumulation of Myeloid-derived Suppressor Cells and Limits Tumor Progression[1]

by

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Running title: Inflammation and tumor-induced MDSC

ABSTRACT

Chronic inflammation is frequently associated with malignant growth and is thought to promote and enhance tumor progression, although the mechanisms which regulate this relationship remain elusive. We reported previously that IL-1β promoted tumor progression by enhancing the accumulation of myeloid-derived suppressor cells (MDSC), and hypothesized that inflammation leads to cancer through the production of MDSC which inhibit tumor immunity. If inflammation-induced MDSC promote tumor progression by blocking anti-tumor immunity, then a reduction in inflammation should reduce MDSC levels and delay tumor progression, while an increase in inflammation should increase MDSC and hasten tumor progression. We have tested this hypothesis using the 4T1 mammary carcinoma and IL-1R-deficient mice which have a reduced potential for inflammation, and IL-1R antagonist (IL-1Ra)-deficient mice, which have an increased potential for inflammation. Consistent with our hypothesis, IL-1R-deficient mice have a delayed accumulation of MDSC and reduced primary and metastatic tumor progression. Accumulation of MDSC and tumor progression are partially restored by IL-6 indicating that IL-6 is a downstream mediator of the IL-1β-induced expansion of MDSC. In contrast, excessive inflammation in IL-1Ra-deficient mice promotes the accumulation of MDSC and produces MDSC with enhanced suppressive activity. These results demonstrate that immune suppression by MDSC and tumor growth are regulated by the inflammatory milieu and support the hypothesis that the induction of suppressor cells which down-regulate tumor immunity is one of the mechanisms linking inflammation and cancer.

INTRODUCTION

Epidemiological and experimental evidence supports the concept that chronic inflammation promotes the development and progression of cancers (1, 2). Since inflammation is a complex process involving many effector cells and mediators, it is likely that inflammation facilitates tumor progression through multiple mechanisms. We have recently proposed that immune suppression may be one of these mechanisms, and have hypothesized that chronic inflammation causes immune suppression which inhibits immune surveillance and/or tumor immunity, thereby enhancing the proliferation of malignant cells (3, 4). This hypothesis was based on findings that chronic inflammation, through the production of the pro-inflammatory cytokine IL-1β, facilitated tumor progression and simultaneously elevated a population of suppressor cells called myeloid-derived suppressor cells ($MDSC^3$) (3, 5). MDSC are a heterogeneous mixture of immature myeloid cells that are potent inhibitors of anti-tumor immunity. They mediate their effects by inhibiting $CD4^+$ and $CD8^+$ T cell proliferation (6-10), by blocking NK cell activation (11, 12), by limiting dendritic cell maturation (13), and by polarizing immunity towards a type 2 phenotype (14). MDSC are found in many patients and experimental animals with cancer (3, 5-11, 13, 15-18), and their induction, expansion, and retention are driven by factors produced by tumor cells and tumor stroma, including potent inflammatory mediators, such as prostaglandin E2 (PGE2) (4) and IL-1β (3, 5).

If inflammation-induced MDSC are a significant factor linking inflammation and cancer, then down-regulating inflammation should reduce MDSC levels and delay tumor progression. We are testing this hypothesis by perturbing the IL-1β signaling pathway in tumor-bearing mice and thereby manipulating the extent of inflammation. The agonists IL-1β and IL-1α bind to the

IL-1 receptor (IL-1R) to induce inflammation, while the IL-1 receptor antagonist (IL-1Ra), attenuates inflammation by inhibiting signal transduction through the IL-1R (19). The balance between IL-1β and IL-1Ra plays an important role in normal physiology, and a disruption of this balance may predispose to or enhance disease, as seen in chronic inflammatory bowel disease (20). An imbalance between IL-1β and IL-1Ra may be due to either overproduction of IL-1β or a deficiency in IL-1Ra, both leading to increased inflammation, as mice deficient for the IL-1Ra show a propensity for spontaneous inflammation (21). In contrast, deletion of the IL-1R reduces inflammation as shown by an increased susceptibility to infection with *L. monocytogenes* and the absence of IL-6, a downstream mediator of IL-1β (22).

We now demonstrate that IL-1R-deficient mice with 4T1 mammary carcinoma tumors have a delayed accumulation of MDSC and slower growing tumors as compared to wild type 4T1-tumor-bearing mice. Conversely, MDSC in tumor-bearing mice deficient for the IL-1Ra accumulate more rapidly and are more potent suppressor cells. Since IL-6 is a key downstream mediator of the IL-1β-induced inflammation pathway, we also demonstrate that IL-6 restores the early accumulation of MDSC and facilitates tumor progression in IL-1R-deficient mice. These findings support the hypothesis that inflammation promotes malignant cell growth by inducing immune suppression, and demonstrate that decreased accumulation of MDSC is one of the mechanisms by which reducing inflammation delays tumor progression.

MATERIAL AND METHODS

Mice. BALB/c mice were purchased from The Jackson Laboratory (Bar Harbor, ME). TS1 and clone 4 mice on a BALB/c background and transgenic for T cell receptors (TCR) reactive to influenza hemagglutinin (HA) peptide 110-119 restricted to I-E^d (23) and peptide 518-526 restricted to K^d (24, 25), respectively, were provided by Dr. E. Fuchs (John Hopkins University, Baltimore, MD). IL-$1R^{-/-}$ (22) and IL-$1Ra^{-/-}$(26) mice, backcrossed to BALB/c mice for 10-12 generations were provided by Drs. M. Kopf (Swiss Federal Institute of technology, Switzerland) and J. Stuart (University of Tennessee Health Science Center, Memphis, TN), respectively. Transgenic and knockout mice were bred in the University of Maryland Baltimore County (UMBC) animal facility. Female mice less than 6 months of age were used for all experiments. All animal procedures were approved by the UMBC Institutional Animal Care and Use Committee.

Plasmids and nucleofection. 4T1 cells were transfected with the BCMG/Neo/hIL-6 plasmid containing the human IL-6 gene (27) (4T1/IL-6) using an Amaxa nucleofector instrument (Gaithersburg, MD), the Nucleofector Solution V and protocol T-24, according to the manufacturer's protocol. Following transfection, cells were cultured for 48 hours, washed with sterile PBS, and placed on G418 selection. 4T1/IL-6 transfectants stably secrete 1.5ng/ml/$5x10^5$/24h of human IL-6 (4T1/IL-6) as quantified by ELISA.

Cell lines. The 4T1 mammary carcinoma and 4T1/IL-1β cell lines were maintained as described (3, 28). The 4T1/IL-6 transfectants were grown in the same medium as the parental cells supplemented with 400 μg/ml G418 (Sigma/Aldrich).

Reagents and antibodies. $HA_{110-119}$ and $HA_{518-526}$ peptides were synthesized in the Biopolymer Core Facility at the University of Maryland, Baltimore (UM,B). Monoclonal antibodies Gr1-PE, CD11b-FITC, CD126-PE (IL-6R), rat IgG2α-PE isotype control and rat IgG2α-FITC isotype control were from BD Pharmingen (San Diego, CA). The arginase inhibitor, N^{w}-hydroxy-nor-L-arginine (nor-NOHA), and the nitric oxide (NO) inhibitor, N^{G}-monomethyl-L-arginine (L-NMMA) were from Calbiochem (San Diego, CA).

IL-6 ELISA and cytokine analysis. Supernatants from 5 x 10^5 cells cultured in 3 ml of media for 24 h were frozen at -80°C until assayed using a hIL-6 DuoSet ELISA kit according to the manufacturer's instructions (R&D Systems). Plates were read at 420 nm on a Bio-Tek 311 microplate reader and quantified using a standard curve. Data are the mean ± SD of triplicate wells. Multiplex cytokine analysis for IFNγ, IL-12p70, MCP-1, IL-6, IL-1β, TNFα and TGFβ was performed by the Cytokine Core Laboratory at UM,B.

Tumor inoculations, tumor tissue dissections and metastasis assay. Tumor inoculations and tumor measurements were as described previously (3, 28). Briefly, female 6-10 week old BALB/c, IL-$1R^{-/-}$, or IL-$1Ra^{-/-}$ mice were inoculated in the mammary fat pad with 7x10^3 tumor cells in 50 μl of PBS. Mice were euthanized when moribund or at the indicated time points. For tumor tissue dissections, primary tumors were surgically removed when tumors were 8-10 mm in

diameter. The tumor tissue was minced with scissors and teased apart, incubated at 37°C in Dulbecco's Modified medium (DMEM) for 24 hours, and supernatants were collected for cytokine analysis. For metastasis assays, lungs were harvested at the indicated times and metastatic disease was quantified using the clonogenic assay by plating cells in 6-thioguanine supplemented media (28).

Blood MDSC. Blood $Gr1^+CD11b^+$ MDSC were obtained as previously described (4). Briefly, mice were tail bled and the percentage of $Gr1^+CD11b^+$ cells was analyzed by flow cytometry. If >90% of the white blood cells were $Gr1^+CD11b^+$, then the mice were euthanized, blood was collected, and RBCs were lysed. The resulting cells were >90% $Gr1^+CD11b^+$ and were used in the subsequent assays.

T cell proliferation assays. The $CD8^+$ and $CD4^+$ T cell proliferation assays were performed as described (6). Briefly, TS1 or clone 4 splenocytes were co-cultured with their respective HA peptide and irradiated blood MDSC from BALB/c, IL-$1R^{-/-}$ or IL-$1Ra^{-/-}$ mice inoculated with 4T1 tumor cells. Cultures were pulsed with ^{3}H-thymidine on day 4, harvested 24 hours later, and the samples counted by scintillation counter. Arginase or NO inhibitors were added to some wells. Data are expressed as the average ± S.D. of triplicate wells.

Flow cytometry. Cells were labeled for direct immunofluorescence as described (28) and analyzed on an Epics XL flow cytometer using Expo32 ADC software (Beckman Coulter, Fullerton, CA).

Bone marrow chimeras. IL-1R$^{-/-}$ mice containing BALB/c bone marrow (BALB/c → IL-1R$^{-/-}$) and BALB/c mice containing IL-1R$^{-/-}$ bone marrow (IL-1R$^{-/-}$ → BALB/c) were constructed as described (29). Briefly, femurs of donor mice were flushed with PBS using a 30-ml syringe fitted with a 27-gauge needle. Bone marrow cells were washed twice with PBS and resuspended in RPMI medium at 200 µl per donor mouse. Recipient mice were lethally irradiated at 850 rads and bone marrow (one donor for two recipients) was inoculated into recipient mice through the tail vein using a 1-ml syringe fitted with a 27-gauge needle. Reconstituted mice received daily injections of gentamicin sulfate i.p. (100 µl of 5 mg/ml) for 7 days beginning 1 day before bone marrow reconstitution. Reconstituted mice were maintained on 0.02% tetracycline drinking water starting 1 week before bone marrow transfer and continuing for 6–8 weeks after reconstitution. Six to eight weeks after bone marrow reconstitution, chimeras were bled from the tail vein, and tested by PCR to ascertain hemopoietic genotype and reconstitution. PCR primers IL-1R-s 5'ATTCTCCATCATCTCTGCTGGTA and IL-1R-as 5' ATCTCAGTTGTCAAGTGTGTCCC were used to detect the ~350 bp amplicon of the IL-1R gene, present only in wild-type BALB/c mice. PCR primers Neo-s 5' TGAATGAACTGCAGGACGAGGCA and Neo-as 5' TCAGCCCATTCGCC GCCAAGCTC were used to detect the 543 bp amplicon of the neomycin insertion gene, present only in the IL-1R$^{-/-}$ mice. PCR annealing temperatures were 56°C and 63°C, respectively, for 30 cycles.

Statistical analyses. Student's two-tailed *t* test for unequal variance was performed using Microsoft Excel 2003. For Figures 2 and 6A, differences in tumor diameter in the inoculated mice were tested using a repeated measures profile analysis of variance (30, 31) using the procedure in SAS V 9.1 (SAS Institute Inc.).

RESULTS

Tumor-associated inflammation is reduced in IL-1R$^{-/-}$ mice

Tumor progression is often accompanied by the presence of inflammation (2). To establish if inflammation accompanies growth of the 4T1 mammary carcinoma, we examined the production of inflammatory cytokines that are hallmarks of inflammatory responses (1, 2). BALB/c mice were inoculated in the abdominal mammary gland with 4T1 tumor cells, primary tumors were removed when tumors reached 8-10 mm in diameter, and the presence of inflammatory cytokines in dissociated 4T1 tumor tissue was compared to normal mammary tissue by multiplex analysis (**Figure 1A**). Canonical pro-inflammatory cytokines, such as IL-6, MCP-1, TGFβ and IL-1β were significantly elevated in 4T1 tumor tissue. The presence of other cytokines associated with inflammation, such as IFNγ and IL-12p70, typically secreted by activated T cells and macrophages, respectively, were also significantly higher in 4T1 tumor tissue. These results demonstrate that the growth of 4T1 primary tumor is associated with an inflammatory microenvironment.

Since IL-1β and several cytokines induced by IL-1β are significantly elevated in 4T1 tumor tissue, we questioned whether a deficiency in the IL-1 receptor (IL-1R) would limit tumor-associated inflammation. BALB/c and BALB/c IL-1R$^{-/-}$ mice were inoculated with 4T1 cells, and tumors were removed when they were 8-10 mm in diameter and analyzed by multiplex analysis for pro-inflammatory cytokines **(Figure 1B)**. Cytokines typically induced by IL-1β, such as IL-6, TNFα and MCP-1 (19), were significantly reduced in the tumor tissue from IL-1R$^{-/-}$ mice compared to tumor tissue from wild type BALB/c mice. Additionally, IL-1R$^{-/-}$ tumor tissue had less IL-12p70 and more TGFβ and IFNγ than tumor tissue from wild type BALB/c

mice. Therefore, loss of IL-1 activity leads to a reduction in inflammation in the tumor and significantly alters the tumor microenvironment.

Tumor progression is delayed in IL-1R-deficient mice

If inflammation drives tumor progression, then tumors may grow more slowly in IL-1R$^{-/-}$ mice as compared to BALB/c mice. To test this hypothesis, BALB/c and IL-1R$^{-/-}$ BALB/c mice were inoculated with 4T1 cells on day 0 and tumor growth was followed. For both groups, tumors were palpable by day 10, and tumor diameters were measured at the indicated time points (**Figure 2A**). The time of tumor onset did not differ between BALB/c and IL-1R$^{-/-}$ mice; however, tumor progression was significantly delayed in IL-1R$^{-/-}$ mice as compared to BALB/c mice ($p<0.05$). These data demonstrate that the absence of IL-1 signaling and a reduction in inflammation in the tumor environment reduce the growth of 4T1 primary tumors.

4T1 tumor progression does not require the IL-1 receptor antagonist

The IL-1 receptor antagonist, IL-1Ra, dampens IL-1β signaling by competing with IL-1β for the IL-1 receptor. Its presence therefore reduces the ensuing inflammatory response, and its absence enhances inflammation (18). Since both the receptor agonist (IL-1β) and the receptor antagonist (IL-1Ra) signal through the IL-1R, the decrease in tumor growth rate in IL-1R$^{-/-}$ mice could be due to the absence of either IL-1β or IL-1Ra. To determine which ligand is responsible, BALB/c and IL-1Ra$^{-/-}$ BALB/c mice were inoculated with 4T1 on day 0 and tumor diameters were measured at the indicated time points (**Figure 2B**). No difference in tumor onset or rate of tumor progression was observed between BALB/c and IL-1Ra$^{-/-}$ mice. Therefore, loss of the IL-

1Ra does not alter tumor growth, indicating that reduced tumor growth in IL-1R$^{-/-}$ mice is due to the absence of IL-1β.

IL-1 non-responsiveness in either hemopoietic or non-hemopoietic cells is sufficient for delayed tumor progression

IL-1β mediates its effects on a variety of cells. To determine if its ability to promote tumor growth involves hemopoietic and/or non-hemopoietic cells, bone marrow chimeras were created. IL-1R$^{-/-}$ or BALB/c mice were lethally irradiated and reconstituted with BALB/c or IL-1R$^{-/-}$ bone marrow to generate BALB/c→IL-1R$^{-/-}$ and IL-1R$^{-/-}$→BALB/c chimeras, respectively. The resulting chimeras were inoculated with 4T1 cells and primary tumor growth was measured weekly **(Figure 2C)**. 4T1 tumor growth was significantly delayed in both BALB/c→IL-1R$^{-/-}$ and IL-1R$^{-/-}$→BALB/c chimeras relative to wild-type BALB/c mice ($p<0.01$ for both); however tumor progression in the chimeras was not significantly different from tumor growth in IL-1R$^{-/-}$ mice. Therefore, IL-1 non-responsiveness in either the hemopoietic or non-hemopoietic cells is sufficient for delayed tumor growth.

IL-1R$^{-/-}$ mice have reduced numbers of lung metastases

Since loss of the IL-1 receptor slows tumor progression we examined whether the absence of the IL-1R also reduces the development of lung metastases. BALB/c and IL-1R$^{-/-}$ mice were inoculated on day 0 with 4T1 tumor cells and mice were sacrificed on day 39-40 when the BALB/c mice were moribund. Lungs were harvested and the number of lung metastases was quantified using the clonogenic assay (28). Lung metastases were significantly reduced ($p<0.05$) in IL-1R$^{-/-}$ mice as compared to BALB/c mice **(Figure 3)**, indicating that the

absence of IL-1 signaling limits metastatic dissemination to the lungs. Therefore, reduced inflammation in the tumor microenvironment is associated with diminished metastatic potential.

The experiment of Figure 2B demonstrated that delayed tumor progression in IL-1R$^{-/-}$ mice was due to a deficiency in signaling by IL-1β rather than signaling by IL-1Ra. To determine whether the reduction in lung metastases in the IL-1R$^{-/-}$ mice was also due to the absence of signaling by IL-1β, BALB/c and IL-1Ra$^{-/-}$ mice were inoculated on day 0 with 4T1 tumor cells and the number of lung metastases was determined on day 39-40. No differences in the number of lung metastases were observed between BALB/c and IL-1Ra$^{-/-}$ mice **(Figure 3)**, indicating that the decrease in lung metastasis in IL-1R$^{-/-}$ mice is due to loss of IL-1β, and not IL-1Ra, signaling.

Since IL-1β mediates tumor progression through both hemopoietic and non-hemopoietic cells **(Figure 2C)**, we examined which cellular compartment regulates the effects of IL-1β on the development of lung metastases. IL-1R$^{-/-}$→BALB/c and BALB/c→IL-1R$^{-/-}$ chimeras were inoculated with 4T1 tumor cells on day 0 and the lungs were harvested on day 39-40. Lung metastases were significantly reduced in the IL-1R$^{-/-}$ group compared to the BALB/c group; however, neither chimera group had significantly fewer lung metastases compared to BALB/c mice (data not shown). Therefore, both hemopoietic and non-hemopoietic cells respond to IL-1β to promote metastatic disease.

Inhibition of IL-1 signaling delays MDSC accumulation

We (3) and others (5) have previously demonstrated that immune suppression driven by MDSC is accentuated by IL-1β-induced inflammation. To determine if the elimination of signaling through IL-1β blocks MDSC accumulation, we examined the levels of MDSC in IL-1R$^{-/-}$, wild type BALB/c, and IL-1Ra$^{-/-}$ mice following inoculation with either 4T1 or 4T1/IL-1β

tumor cells. Mice were inoculated on day 0 and the percent of $CD11b^{+}Gr1^{+}$ MDSC in the blood was measured at various time points by flow cytometry **(Figure 4)**. MDSC levels in BALB/c mice with 4T1/IL-1β tumors and in $IL\text{-}1Ra^{-/-}$ mice with 4T1 tumors increased significantly by day 8-10 compared to BALB/c mice with parental 4T1 tumors ($p<0.05$). In contrast, MDSC accumulation was significantly delayed in $IL\text{-}1R^{-/-}$ mice and did not reach the levels of MDSC in BALB/c mice until days 26-28. By days 26-40, MDSC levels were >80% of the blood cells in all groups of mice. Therefore MDSC accumulate more slowly in the early stages of tumor growth in mice that are not responsive to IL-1β.

To determine whether MDSC accumulation is driven by IL-1 responsiveness of host hemopoietic or non-hemopoietic cells, bone marrow chimeric mice were inoculated with 4T1 tumor cells and blood MDSC were measured by flow cytometry. Percentages of MDSC in the BALB/c→$IL\text{-}1R^{-/-}$ chimeras and in non-chimeric $IL\text{-}1R^{-/-}$ mice were not significantly different; and both BALB/c→$IL\text{-}1R^{-/-}$ and $IL\text{-}1R^{-/-}$→BALB/c chimeras had significantly reduced levels of MDSC compared to BALB/c mice ($p<0.05$) (data not shown). Therefore, a deficiency for the IL-1R in either the hemopoietic or non-hemopoietic compartment is sufficient to delay MDSC accumulation.

Reduced inflammation limits the quantity of MDSC but does not alter the suppressive quality of MDSC

The delay in MDSC accumulation in $IL\text{-}1R^{-/-}$ mice demonstrates that a reduction in inflammation alters the quantity of MDSC. We have previously reported that MDSC induced by 4T1/IL-1β tumors are more suppressive than MDSC induced by parental 4T1 tumors (3). If, as these previous experiments suggested, an enhanced pro-inflammatory cytokine milieu affects

both the quantity and the quality of MDSC, then MDSC from IL-1R-deficient mice may be less suppressive than MDSC from IL-1β-competent mice. To determine the role of IL-1β in MDSC activity, BALB/c and IL-1R$^{-/-}$ mice were inoculated with 4T1 tumor cells on day 0 and blood MDSC were harvested when MDSC were >90% of cells in the blood (days 35-40). Suppressive activity was tested by co-culturing splenocytes from TS1 mice with their respective HA peptide in the presence or absence of graded doses of MDSC from BALB/c or IL-1R$^{-/-}$ mice (**Figure 5A**). MDSC from BALB/c and IL-1R$^{-/-}$ mice suppressed CD4^{+} T cell proliferation by >90% as measured by ^{3}H-thymidine uptake. To identify the mechanism of suppression, inhibitors of arginase or NO, nor-NOHA or L-NMMA, respectively, were added to the TS1 cultures. The addition of nor-NOHA completely reversed the suppression of both BALB/c and IL-1R$^{-/-}$ MDSC, while the addition of L-NMMA had no effect. These data demonstrate that although the loss of IL-1 and a reduction in the inflammatory environment limits the quantity of MDSC, it does not alter the functional activity of MDSC.

IL-1Ra$^{-/-}$ MDSC are more suppressive than BALB/c MDSC

Previous studies demonstrated that MDSC from BALB/c mice with 4T1/IL-1β tumors accumulate earlier and at a faster rate, and are a functionally more suppressive population of MDSC (3). If the loss of the IL-1Ra enhances inflammation and promotes MDSC accumulation, then IL-1Ra$^{-/-}$ MDSC may be functionally more suppressive than BALB/c MDSC. To test this hypothesis, BALB/c and IL-1Ra$^{-/-}$ mice were inoculated with 4T1 tumor cells on day 0, and blood MDSC were harvested when MDSC were >90% of the cells in the blood (days 35-40).

Suppressive activity was tested by co-culturing splenocytes from TS1 mice with their respective HA peptide in the presence or absence of graded doses of MDSC. MDSC from IL-1Ra$^{-/-}$ mice were more suppressive towards CD4^{+} TS1 cells on a per cell basis than MDSC from BALB/c mice (**Figure 5B**). MDSC from 4T1-inoculated BALB/c and IL-Ra-deficient mice were also tested for suppressive activity against CD8^{+} T cells using splenocytes from clone 4 mice and their respective HA peptide **(Figure 5C)**. Similar to the results with CD4^{+} T cells, MDSC from IL-1Ra$^{-/-}$ mice were more suppressive on a per cell basis than MDSC from BALB/c mice ($p<0.05$). To determine if MDSC from IL-1Ra-deficient mice suppress by the same arginase-dependent mechanism as MDSC derived from BALB/c mice (3, 6), an inhibitor of arginase (nor-NOHA) or nitric oxide (L-NMMA) was added to the TS1 or clone 4 cultures **(Figures 5B and 5C)**. MDSC from BALB/c and IL-1Ra$^{-/-}$ mice suppressed CD4^{+} and CD8^{+} T cells through an arginase-dependent mechanism, as addition of the arginase inhibitor, but not the NO inhibitor, reversed MDSC mediated suppression. Therefore, elimination of the IL-1Ra induced MDSC that are more potent suppressors but use the same suppressive mechanism as MDSC induced by wild-type mice.

IL-6 compensates for the loss of the IL-1 receptor in primary tumor growth

The reduction in tumor growth observed in the IL-1R$^{-/-}$ mice may be due to the loss of the direct effects of IL-1β or may be due to the loss of a downstream product of the IL-1 signaling pathway. A potential downstream candidate is the pro-inflammatory cytokine, IL-6, whose production is reduced in IL-1R$^{-/-}$ tumor tissue **(Figure 1B)**. To examine whether IL-6 is involved in IL-1-mediated tumor progression, 4T1 tumor cells were stably transfected with a construct containing the human IL-6 gene to generate 4T1/IL-6 cells. Because murine cells are responsive

to human IL-6 (32), the human IL-6 gene was used to differentiate between IL-6 secreted by the 4T1/IL-6 cells and endogenous murine IL-6 present in the serum and tumor environment. 4T1/IL-6 cells have the same in vivo growth rate as 4T1 cells, so human IL-6 is not serving as an alloantigen **(Figure 6A)**. If IL-6 is a downstream mediator by which IL-1β alters MDSC activity and tumor progression, then IL-6 production should restore elevated MDSC levels and more rapid tumor progression in IL-1R-deficient mice. To test this hypothesis, IL-1R$^{-/-}$ and BALB/c mice were inoculated with 4T1/IL-6 tumor cells and tumor progression was monitored. No delay in tumor growth was observed in IL-1R$^{-/-}$ mice bearing 4T1/IL-6 tumors compared to the significant delay seen in IL-1R$^{-/-}$ mice with 4T1 tumors **(Figure 6A)**. Additionally, 4T1/IL-6 tumors in IL-1R$^{-/-}$ mice grew at a similar rate to 4T1 and 4T1/IL-6 tumors in BALB/c mice. Therefore, IL-6 compensates for the loss of IL-1R, supporting the hypothesis that IL-6 is a downstream mediator of IL-1β that facilitates tumor progression.

IL-6 compensates for the loss of IL-1R in the development of lung metastases

Since IL-6 restores primary tumor growth in IL-1R-deficient mice, we examined whether IL-6 also eliminates the reduction in lung metastases. BALB/c and IL-1R$^{-/-}$ mice were inoculated with 4T1/IL-6 tumor cells on day 0 and sacrificed on day 39-40. Lung metastases were quantified as previously described and compared to the levels of lung metastases in BALB/c and IL-1R$^{-/-}$ mice inoculated with 4T1 parental cells **(Figure 6B)**. IL-6 restores the number of lung metastases in IL-1R-deficient mice to that seen in BALB/c mice, demonstrating that IL-6 may be a downstream mediator of IL-1 for metastatic dissemination to the lungs.

IL-6 restores MDSC accumulation in IL-1R$^{-/-}$ mice

The delay in MDSC accumulation in IL-1R$^{-/-}$ tumor-bearing mice and the IL-1β-induced induction of MDSC suggest that inflammation through the production of IL-1β promotes the accumulation of MDSC and contributes to tumor progression. However, MDSC do not express the IL-1R (3) and therefore can not respond directly to IL-1β. Therefore, it is likely that IL-1β indirectly affects MDSC activity via mediators downstream of IL-1β. Because IL-6 compensates for the loss of IL-1 with respect to primary and metastatic tumor growth, we examined whether IL-6 might be a downstream mediator that affects MDSC. If IL-6 is a downstream mediator, then secretion of IL-6 by 4T1 tumor cells could compensate for the delay in MDSC accumulation in IL-1R-deficient mice. To test this possibility, BALB/c and IL-1R$^{-/-}$ mice were inoculated with 4T1 or 4T1/IL-6 tumor cells at day 0, periodically tail bled, and the percentage of blood MDSC determined by flow cytometry **(Figure 6C)**. On days 14-16 when the delay in MDSC in IL-1R$^{-/-}$ is most pronounced, there is no observed delay in IL-1R$^{-/-}$ mice inoculated with 4T1/IL-6 tumor cells, suggesting that IL-6 production compensates for the loss of IL-1 signaling.

IL-6 could be acting directly on MDSC or it could trigger other downstream factors that directly act on MDSC. For a direct effect, MDSC would need to express the receptor for IL-6. To determine if MDSC express the IL-6 receptor, MDSC from BALB/c mice with 4T1 or 4T1/IL-1β tumors, and MDSC from IL-1R$^{-/-}$ mice with 4T1 tumors were stained for the IL-6R and analyzed by flow cytometry **(Figure 6D)**. MDSC uniformly express the IL-6R whether they are derived from BALB/c or IL-1R$^{-/-}$ mice or induced by 4T1 or 4T1/IL-1β tumor cells. Therefore, MDSC have the capability to directly respond to IL-6.

DISCUSSION

Although the concept is widely accepted, the pathways by which inflammation lead to tumor progression remain elusive. We have proposed that one of the contributing mechanisms may be the induction of myeloid-derived suppressor cells that limit anti-tumor immunity and thereby promote tumor growth (3, 4). This hypothesis was based on studies in which inflammation was exacerbated by increasing pro-inflammatory mediators. Although these studies showed a strong correlation, they did not eliminate the possibility that immune suppression simply accompanies inflammation, rather than being caused by inflammation. Using two very different experimental approaches to alter the inflammatory milieu, we now report that reducing inflammation by blocking signaling through the IL-1R, or increasing inflammation through loss of the IL-1Ra, alters the accumulation of MDSC and tumor progression in agreement with the concept that inflammation promotes tumor progression by activating immune suppressive networks.

Interestingly, the increase in MDSC is dramatic during early tumor growth, and diminishes at later stages, indicating that the effect of inflammation on MDSC accumulation is an early event in tumor progression. Since many of the factors that produce MDSC are also secreted by tumor cells (e.g. IL-6, IL-10, VEGF) (1, 2, 33) the bolus induction of MDSC by inflammation may only be detectable at the early stages because as tumors increase in mass they are producing proportionately more MDSC induction factors which over-ride the local inflammatory environment. Alternatively, the effects of inflammation may only be detectable early because MDSC levels reach their maximum at later stages and there is no “space” for additional MDSC in the blood or spleen. Indeed, many of the agents known to induce MDSC

are associated with and/or induced by inflammation, suggesting that the inflammatory environment of progressively growing tumors promotes MDSC accumulation through multiple effector molecules. For example, VEGF, an essential molecule for neo-angiogenesis, is induced by inflammation (1) and is correlated with the presence of MDSC in the peripheral blood (34, 35). Likewise, IL-6 (33, 36, 37), another pro-inflammatory mediator, and PGE2 (4, 38), a product of inflammation, induce MDSC accumulation. MDSC may also enhance inflammation as their production of VEGF, IL-10 and reactive oxygen species, which mediate their suppressive function, may contribute to the inflammatory milieu (14, 33, 35). Whether there are MDSC that are induced by inflammation-independent factors, or whether the extent of inflammation governs the quality and quantity of MDSC is unclear. The studies reported here using the IL-1Ra knockout mice and earlier studies using IL-1β-secreting tumor cells (3) demonstrate that heightened inflammation induces more MDSC with more suppressive activity. These observations do not necessarily indicate that inflammation induces a novel population of MDSC, because the tumor microenvironment itself is an inflammatory microenvironment and the added inflammation could simply be an amplification effect. However, the quantity and quality of MDSC may be differentially regulated, since excessive inflammation, through tumor cell production of IL-1β or loss of the IL-1Ra, increases both the quantity and the suppressive activity of MDSC, while a reduction in inflammation, as seen in the IL-1R$^{-/-}$ mice, only affects MDSC quantity. Further complicating this issue, is the observation that MDSC are not a homogeneous population of cells, but are a heterogeneous mixture of immature myeloid cells. Indeed, inflammation and non-inflammatory mediators may impact subpopulations of MDSC differently.

As shown in previous studies, IL-1β induces the expansion of MDSC indirectly as MDSC do not have the IL-1R (3). This earlier observation led us to examine other potential downstream mediators of IL-1β for their effects on the accumulation of MDSC. IL-6 was a prime candidate because it has previously been implicated in the accumulation of MDSC (33), and because hyperactivation of its transcription factor, STAT3, promotes abnormal differentiation of DC (37). Our finding that MDSC express the IL-6R and that tumor-secreted IL-6 at least partially restores MDSC accumulation and enhanced tumor progression in IL-1R-deficient mice, confirms that IL-6 is likely to be a relevant IL-1β downstream mediator. Although tumor-secreted IL-6 increases tumor growth and MDSC levels in IL-1R$^{-/-}$ mice, it does not fully restore enhanced tumor growth or MDSC accumulation to that seen in wild type mice with 4T1/IL-1β tumors. There are several possibilities for this apparent discrepancy. The quantity of IL-6 produced by the transfectants may not be equivalent to the in vivo amount of IL-6 induced by the IL-1β transductants. Alternatively, IL-6 may be only one of several downstream mediators induced by IL-1β, and maximal induction of MDSC and tumor growth may require additive or synergistic action with other downstream mediators. Previous studies demonstrated that PGE2 also induces the accumulation of MDSC and hastens tumor progression (4). Since PGE2 is also induced by IL-1β (19), it may be another downstream mediator that acts in conjunction with IL-6.

Novel immune-based therapies for the treatment of cancer are currently under development. Many of these approaches involve active immunization and are likely to be most effective in immunocompetent tumor-bearing individuals who are minimally immunosuppressed. Given the causal relationship between inflammation and the induction of MDSC, adjunctive

therapies that reduce inflammation prior to immunotherapy, may significantly enhance the efficacy of any active immunotherapy.

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FOOTNOTES:

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[3]Abbreviations used in this study:

4T1/IL-1β, 4T1 cells transduced with construct expressing hIL-1β

4T1/IL-6, 4T1 cells transfected with construct expressing hIL-6

HA,	influenza hemagglutinin
IL-1Ra,	interleukin 1 receptor antagonist
IL-1R,	interleukin 1 receptor type I
IL-6R,	interleukin 6 receptor
L-NMMA,	N^G-monomethyl-L-arginine
MCP-1,	monocyte chemoattractant protein
MDSC,	myeloid-derived suppressor cell(s)
Nor-NOHA,	N^W-hydroxyl-nor-L-arginine
PGE2,	prostaglandin E2

FIGURE CAPTIONS

Figure 1. Inflammation-associated cytokines are higher in 4T1 primary tumors of BALB/c mice than in 4T1 tumors of IL-1R-deficient mice. BALB/c and IL-1R$^{-/-}$ mice were inoculated with 7000 4T1 tumor cells in the abdominal mammary gland on day 0, and primary tumors were surgically removed when their diameters reached 8-10 mm. The resulting tumor tissue and control mammary tissue from tumor-free mice was minced, incubated at 37°C for 24 hours, and the resulting supernatants were assayed for cytokines by multiplex analysis. (A) 4T1 tumor tissue vs. normal mammary tissue from BALB/c mice. (B) 4T1 tumor tissue from BALB/c mice compared to 4T1 tumor tissue from IL-1R$^{-/-}$ mice. Values are the average ± SD of triplicates of individual mice.

Figure 2. Tumor progression is delayed in IL-1R$^{-/-}$ mice but not in IL-1Ra$^{-/-}$ mice. (A) BALB/c and IL-1R$^{-/-}$ mice were inoculated with 7000 4T1 tumor cells in the abdominal mammary gland and monitored for primary tumor growth. Tumor progression was delayed in IL-1R$^{-/-}$ mice relative to BALB/c mice ($p<0.05$). (B) 4T1 tumor growth does not differ in BALB/c and IL-1Ra$^{-/-}$ mice. (C) BALB/c, IL-1R$^{-/-}$ and BALB/c→IL-1R$^{-/-}$ and IL-1R$^{-/-}$→BALB/c bone marrow chimeras were inoculated with 4T1 tumor cells and monitored for primary tumor growth. Tumor progression in both chimeras was delayed compared to BALB/c mice ($p<0.05$). Values are the average ± SD of 10-15, 10-15, and 7-15 mice per group for A, B and C, respectively. Data are pooled from 2-3 experiments.

Figure 3. IL-1R$^{-/-}$, but not IL-1Ra$^{-/-}$, mice have reduced lung metastases. BALB/c, IL-1R$^{-/-}$ and IL-1Ra$^{-/-}$ mice were inoculated with 7000 4T1 tumor cells in the abdominal mammary gland on day 0, and their lungs harvested on day 35-40, when BALB/c mice were moribund. Metastatic cells were quantified using the clonogenic assay. Lung metastases are reduced in IL-1R$^{-/-}$ mice relative to BALB/c mice ($p<0.05$). The number of metastatic cells in the lungs of individual mice (●) is shown. Data are pooled from 2-3 experiments.

Figure 4. IL-1β or the absence of the IL-1Ra enhances MDSC accumulation, while the absence of the IL-1R delays MDSC accumulation. BALB/c, IL-1R$^{-/-}$, and IL-1Ra$^{-/-}$ mice were inoculated with 7000 4T1 or 4T1/IL-1β tumor cells in the abdominal mammary gland on day 0. Mice were tail bled at the indicated time points and the percentage of CD11b^{+}Gr1^{+} MDSC was quantified by flow cytometry. MDSC are elevated in BALB/c mice with 4T1/IL-1β tumors and in IL-1Ra$^{-/-}$ mice with 4T1 tumors by day 8, and remain elevated through day 16 ($p=0.05$ and $p<0.05$, respectively for day 8-10; $p<0.01$ for both groups on day 14-16). MDSC are reduced in IL-1R$^{-/-}$ mice ($p<0.01$). Data are the average ± SD of 5-15 mice per group and are pooled from 2 experiments.

Figure 5. MDSC from IL-1Ra$^{-/-}$ mice are more suppressive towards CD4^{+} and CD8^{+} T cells than MDSC from BALB/c mice. BALB/c, IL-1R$^{-/-}$ and IL-1Ra$^{-/-}$ mice were inoculated with 7000 4T1 tumor cells in the abdominal mammary gland, and tail bled to test for CD11b^{+}Gr1^{+} MDSC. When MDSC were >90% of the white blood cells, mice were sacrificed, blood was collected and RBCs were lysed. The resulting MDSC were used in T cell proliferation assays. CD4^{+} TS1 or CD8^{+} clone 4 transgenic splenocytes were stimulated with $HA_{110-119}$ or $HA_{518-526}$ peptide,

respectively, and co-cultured in the presence or absence of graded doses of blood MDSC. Inhibitors of arginase (nor-NOHA) or nitric oxide (L-NMMA) were added to some of the wells. T cell proliferation was measured as cpm of [^{3}H]-thymidine. (A) BALB/c and IL-1R$^{-/-}$ MDSC were equally suppressive. (B and C) IL-1Ra$^{-/-}$ MDSC were more suppressive than BALB/c MDSC towards (B) CD4$^+$ T cells ($p<0.05$) and (C) CD8$^+$ T cells ($p<0.05$). Data are from one of 2-5 independent experiments.

Figure 6. IL-6 compensates for tumor progression and MDSC accumulation in IL-1R$^{-/-}$ mice. BALB/c and IL-1R$^{-/-}$ mice were inoculated with 7000 4T1 or 4T1/IL-1β tumor cells in the abdominal mammary gland on day 0 and monitored for primary tumor growth, lung metastases, and blood MDSC. (A) Tumor diameter was measured at the indicated time points. Values are the average ± SD of 15-17 mice per group. (B) Mice were sacrificed and lungs were harvested when BALB/c mice were moribund (days 35-40). Metastatic cells in individual mice (●) were quantified using the clonogenic assay. (C) The percentage of blood CD11b$^+$Gr1$^+$ MDSC was measured by flow cytometry at the indicated time points. Values are the average ± SD of 15-17 mice per group and are pooled from 3 independent experiments. (D) Blood CD11b$^+$Gr1$^+$ MDSC from tumor-bearing BALB/c or IL-1R$^{-/-}$ mice with 4T1 or 4T1/IL-1β were stained with IL-6R antibody or isotype control antibody. 4T1 tumor cells and BALB/c splenocytes were stained as negative and positive controls, respectively. All MDSC express the IL-6R. Data are pooled from 2-3 experiments.

Figure 1: Inflammation-associated cytokines are higher in 4T1 primary tumors of BALB/c mice than in 4T1 tumors of IL-1R-deficient mice.

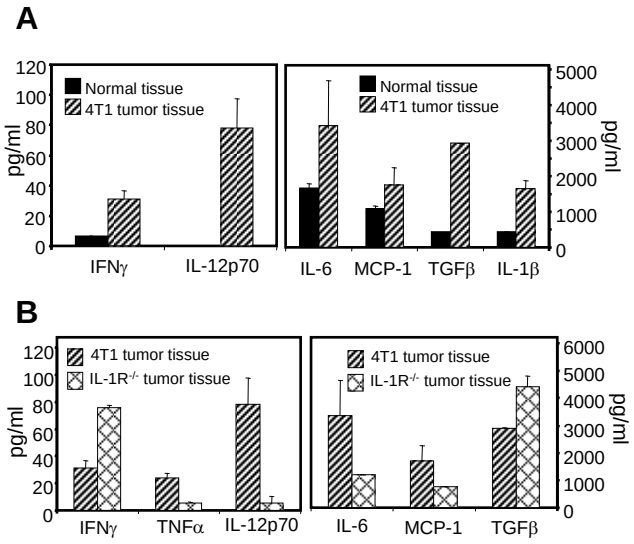


Figure 2: Tumor progression is delayed in IL-1R$^{-/-}$ mice but not in IL-1Ra$^{-/-}$ mice.

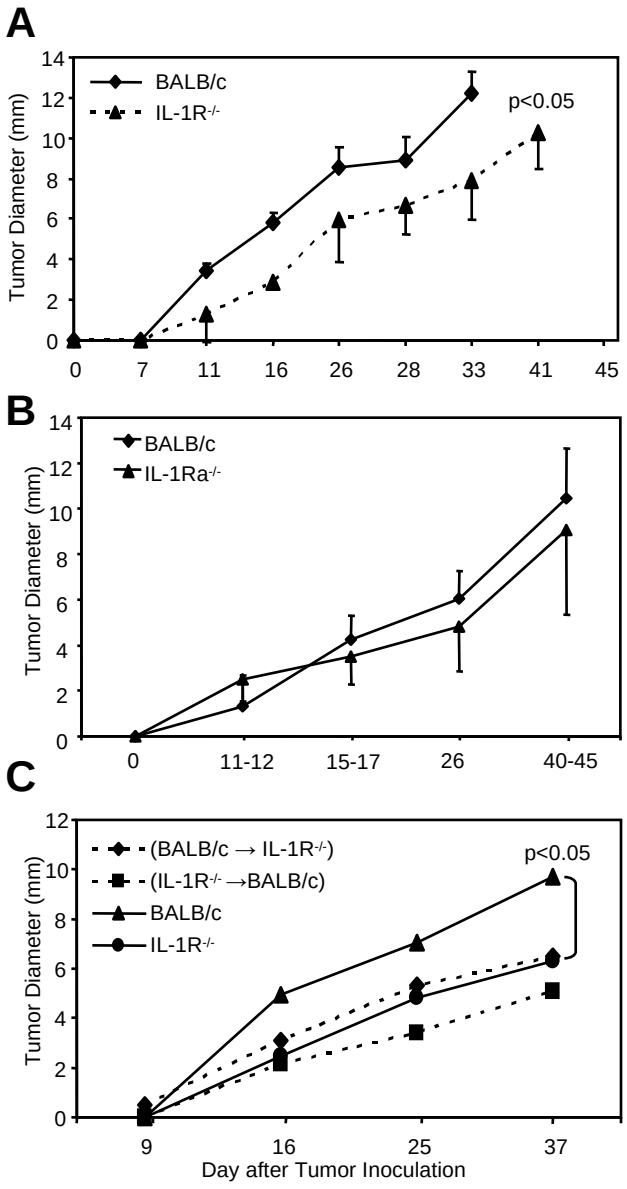


Figure 3: IL-1R$^{-/-}$, but not IL-1Ra$^{-/-}$, mice have reduced lung metastases.

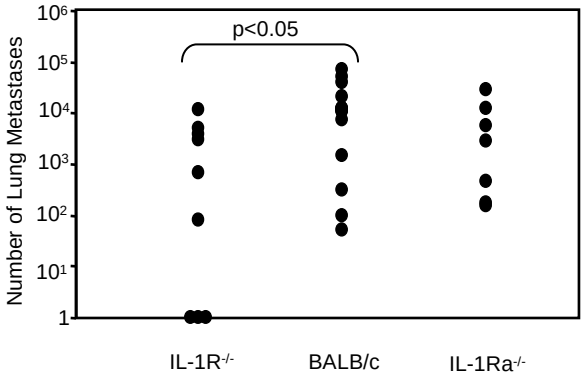


Figure 4: IL-1β or the absence of the IL-1Ra enhances MDSC accumulation, while the absence of the IL-1R delays MDSC accumulation.

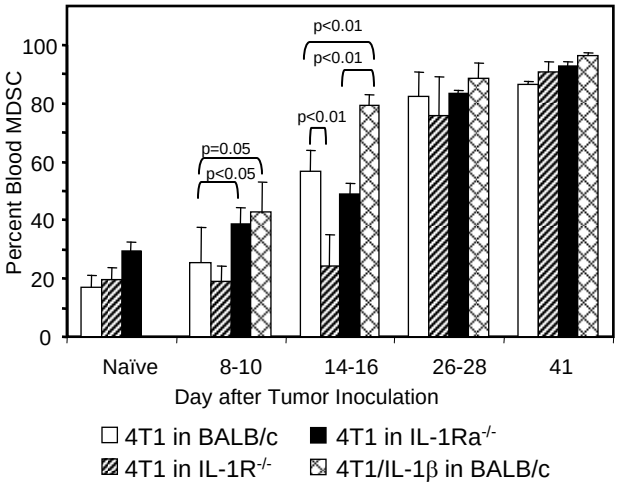


Figure 5: MDSC from IL-1Ra$^{-/-}$ mice are more suppressive towards CD4^{+} and CD8^{+} T cells than MDSC from BALB/c mice.

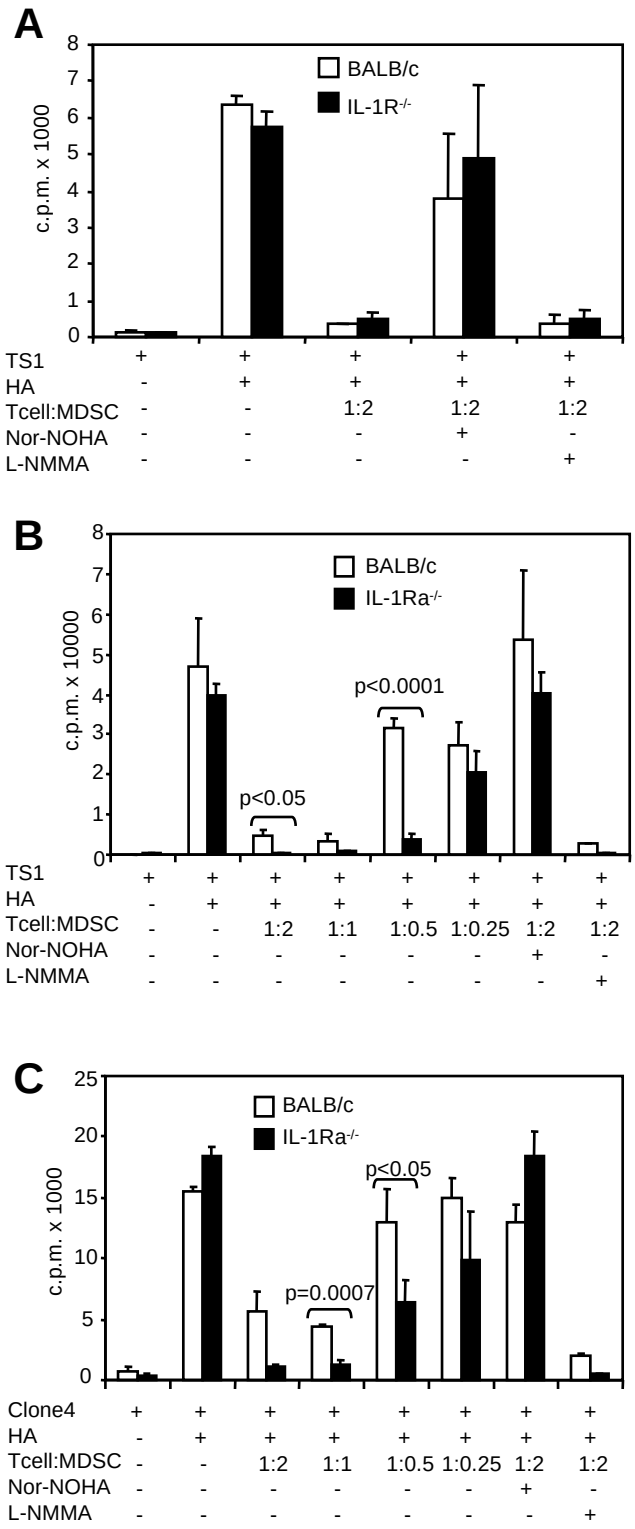
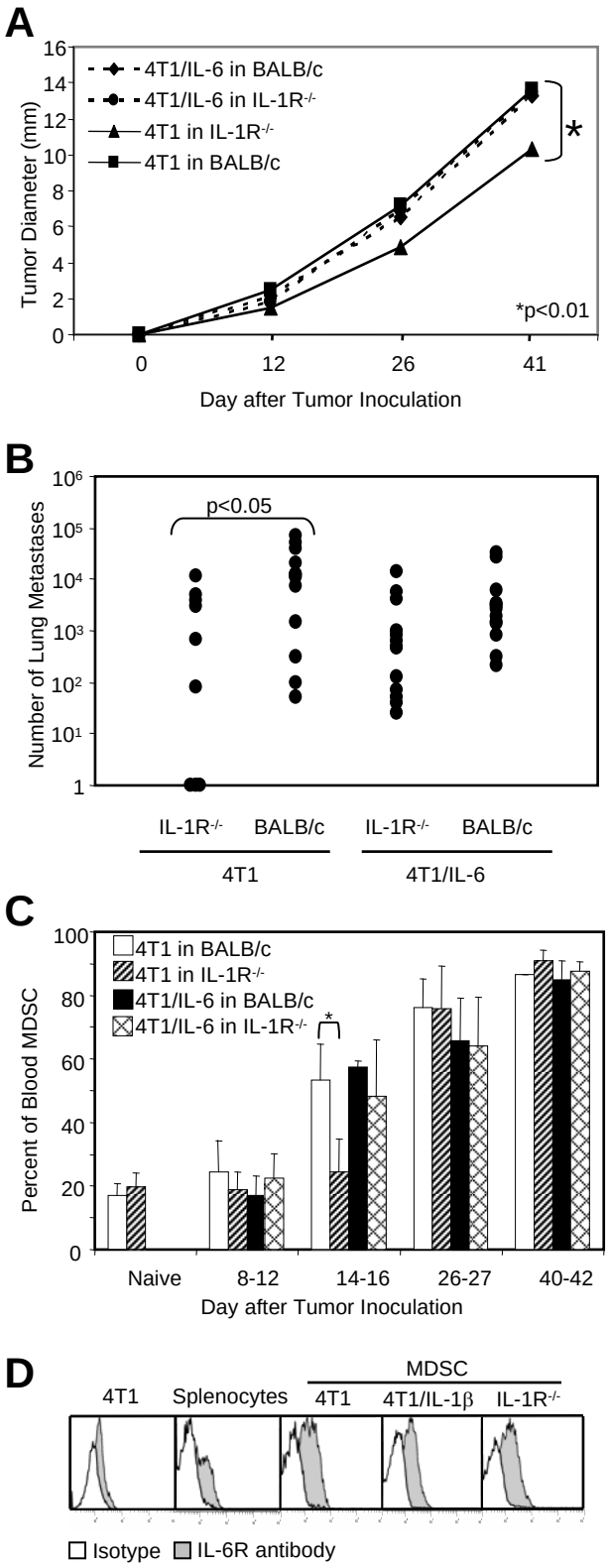


Figure 6: IL-6 compensates from tumor progression and MDSC accumulation in IL-1R$^{-/-}$ mice.



APPENDIX C

Inflammation Induces Tumor Growth by Nuclear-factor-κB Signaling in Myeloid-derived Suppressor Cells

Manuscript in Preparation

Inflammation Induces Tumor Growth by Nuclear-factor-κB Signaling in Myeloid-derived Suppressor Cells

By

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Running title: NFκB activation of myeloid-derived suppressor cells

Key words: tumor-induced immune suppression, inflammation, T cell activation

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ABSTRACT

Myeloid-derived suppressor cells (MDSC) are potent inhibitors of anti-tumor immunity that facilitate tumor progression by blocking the activation of $CD4^+$ and $CD8^+$ T cells and by promoting a type 2 immune response through their production of IL-10 and down-regulation of macrophage production of IL-12. Because they accumulate in many cancer patients, MDSC are a significant impediment to active cancer immunotherapies. Chronic inflammation has recently been shown to enhance the accumulation of MDSC, and to increase their suppression of T cells. These findings led us to hypothesize that inflammation contributes to tumor progression through the induction of MDSC which create a favorable environment for tumor growth. Since chronic inflammation also drives type 2 immune responses which favor tumor growth, we have now asked if inflammation mediates this latter effect through MDSC. We find that inflammation increased IL-10 production by MDSC and induces MDSC that are more effective at down-regulating macrophage production of IL-12 as compared to MDSC isolated from less inflammatory tumor micro-environments, thereby skewing tumor immunity towards a type 2 response. In vitro experiments using bacterial lipopolysaccharide demonstrated that MDSC phenotype is induced by signaling through the TLR4-NFκB pathway, and involves up-regulation of CD14. Although NFκB is well-recognized as a key regulatory protein in other myeloid cells, it has not previously been implicated in MDSC function. These studies demonstrate that MDSC are an intermediary through which inflammation promotes type 2 immune responses, and they identify a novel mechanism by which activation of NFκB down-regulates tumor immunity and enhances tumor growth.

INTRODUCTION

Acute inflammation is a self-limiting, localized response to tissue injury or trauma caused by wounding or infection. This response is aimed at healing the affected tissue by the induction of signals which trigger innate and adaptive immune responses (1). Immune cells, primarily macrophages, dendritic cells (DC), and immature bone-marrow derived cells, respond to inflammatory signals, such as lipopolysaccharide (LPS) from gram-negative bacteria, through activation of the TLR4 and CD14 pathway (2). These acute inflammatory responses typically induce type-1 immunity that eliminates the initiating stimulus and dampens the inflammatory response. However, persistence of the pathogen, dysregulation of the inflammatory response, or the inhibition of anti-inflammatory mechanisms may transform acute responses into chronic inflammation and lead to the development of chronic inflammatory diseases (1, 3). In addition, failure to dampen the inflammatory response is frequently associated with the onset of cancers, and is characterized by a predominantly type-2 immune response, which favors tumor progression (4).

Chronic inflammation is also associated with the induction and promotion of immune suppressive mechanisms, such as the accumulation of myeloid-derived suppressor cells (MDSC), and we have proposed that inflammation may contribute to malignancy by the induction of MDSC (5, 6). MDSC are a heterogenous population of immature myeloid cells that are present in low levels in healthy individuals, and are elevated in patients and experimental animals with cancers (5, 7-15). They function as potent inhibitors of anti-tumor immunity by blocking the activation of $CD4^+$ and $CD8^+$ T cells (7, 9-12), NK cytotoxicity (16, 17), and maturation of DC (8). In contrast to most immature myeloid cells that are matured by inflammatory stimuli to promote type-1 immune responses (2), MDSC maintain an immature phenotype when exposed to pro-inflammatory signals and instead contribute to a tumor-promoting type-2 phenotype by their production of IL-10 and their blocking of

macrophage production of IL-12 (18). If inflammation facilitates tumor progression through the induction of more suppressive MDSC, then an increasingly pro-inflammatory environment may enhance the potency of MDSC. Previous studies have confirmed this hypothesis with respect to T cell activation and have shown that increasing inflammation yields more suppressive MDSC (5, 6). We have now tested the hypothesis that inflammation further inhibits anti-tumor immunity by enhancing the type-2 phenotype of MDSC, and report that inflammation, signaling through the LPS-TLR4-NFκB pathway, heightens the type 2 phenotype of MDSC by increasing MDSC expression of CD14, activation of NFκB, and production of IL-10.

MATERIALS AND METHODS

Mice. Mating pairs of BALB/c and BALB/c TLR4$^{-/-}$ mice were purchased from The Jackson Laboratory (Bar Harbor, ME). Mating pairs of IL-1Ra$^{-/-}$ (19) mice, backcrossed to BALB/c mice for 10-12 generations, were provided by Dr. J. Stuart (University of Tennessee Health Science Center, Memphis, TN). Female mice less than 6 months of age, bred in the University of Maryland Baltimore County (UMBC) animal facility, were used for all experiments. All animal procedures were approved by the UMBC Institutional Animal Care and Use Committee.

Reagents and antibodies. Monoclonal antibodies Gr1-PE, CD11b-FITC, CD11b-PerCP, CD14-FITC, CD80-FITC, CD86-PE, CD11c-PE, rat IgG2α-PE isotype control and rat IgG2α-FITC isotype control were from BD Pharmingen (San Diego, CA), F4/80-FITC was from Invitrogen (Carlsbad, CA), and TLR4-PE was from Biolegend (San Diego, CA). MAbs F4/80-FITC and DEC205-FITC were from Cedarlane (Burlington, Canada) and Invitrogen (Carlsbad, CA), respectively. LPS was from Difco (Detroit, MI) and IFNγ was from Pierce (Rockford, IL).

Cell lines. The 4T1 mammary carcinoma and 4T1/IL-1β cell lines were maintained as described (5, 20).

Tumor inoculations and measurements. Tumor inoculations and tumor measurements were as described previously (5, 20). Briefly, female 6-10 week old BALB/c, TLR4$^{-/-}$, or IL-1Ra$^{-/-}$ mice were inoculated in the mammary fat pad with $7x10^3$ tumor cells in 50 μl of PBS. Mice were euthanized when tumor diameters reached 10-12mm or when mice were moribund.

Blood MDSC. Blood $Gr1^+CD11b^+$ MDSC were obtained as previously described (18). Briefly, mice were tail bled and the percentage of $Gr1^+CD11b^+$ cells was analyzed by flow cytometry. If >85% of the white blood cells were $Gr1^+CD11b^+$, then the mice were euthanized, blood was collected, and RBCs were lysed. The resulting cells were >90% $Gr1^+CD11b^+$ and were used in the subsequent assays.

Peritoneal macrophages. Mice were inoculated i.p. with 1 ml of 3% thioglycolate (Difco, Detroit, MI), and peritoneal exudate cells were collected 5 days later. Greater than 90% of the peritoneal exudates cells were $F4/80^+$ and $CD11b^+$ as measured by flow cytometry.

Macrophage and MDSC co-culture experiments. Peritoneal macrophages from BALB/c or $TLR4^{-/-}$ mice were co-cultured with blood-derived MDSC as previously described (18). Briefly, macrophages were plated at $7.5x10^5$ cells/well in 500 μl of macrophage medium (DMEM supplemented with 10% FBS) in 24 well plates and incubated at 37˚C for 3 hours. Non-adherent cells were removed and 500 μl of 5% FBS in DMEM were added to the adherent cells. $1.5x10^6$ MDSC in 500 μl of 5% FBS in DMEM were added to some wells. Macrophages and MDSC co-cultures were then stimulated with LPS (100ng/ml) and/or IFNγ (2ng/ml) and cultures were incubated at 37˚C for 24 hrs. Culture supernatants were collected and stored at -80^oC until analyzed for cytokines. Cells were detached using a cell scraper, washed with cold sterile PBS, stained with antibodies, and analyzed by flow cytometry.

IL-10 and IL-12 ELISAs. Thawed supernatants from macrophage/MDSC co-cultures were assayed by ELISA for IL-10 and IL-12 using duo set kits (R&D Systems, Minneapolis, MN) according to the

manufacturer's protocols. Plates were read at 420 nm using a Bio-Tek 311 microplate reader and quantified using a standard curve. Data are the mean ± SD of triplicate wells.

Western blots. Blood MDSC and control HELA cells were labeled with p-IκBα and IκBα antibodies using a PhosphoPlus IκB-α antibody kit (Cell Signaling, Danvers, MA). Blood MDSC were re-suspended to $1x10^7$ cells/ml in lysis buffer (20 mM Tris (pH 7.5), 150 mM NaCl, 1mM EDTA, 1% Triton X-100, 2.5 mM sodium pyrophosphate, 1 mM Na_3VO_4, 0.5% Nonidet P-40, 50 mM NaF, and 10X protease inhibitor cocktail), incubated on ice for 30 min, and centrifuged for 15 min. The resulting supernatants were frozen at -20°C until used. Western blots and antibody staining were performed according to the manufacturer's protocol. A β-actin monoclonal antibody (1:10000; Sigma, St. Louis, MO) was used to control for protein loading. Briefly, lysates were electrophoresced on 12% SDS-PAGE gels and transferred to Hybond-P membranes (Amersham, Piscataway, NJ) using a Bio-Rad Mini Trans-blot cell at 100V for 75 min. Membranes were blocked in 5% milk and stained in 5% BSA.

Flow cytometry. Cells were labeled by direct immunofluorescence as described (20) and analyzed on an Epics XL flow cytometer using Expo32 ADC software (Beckman Coulter, Fullerton, CA).

Statistical analyses. Student's two-tailed *t* test for unequal variance was performed using Microsoft Excel 2003.

RESULTS

Inflammation promotes MDSC suppression of macrophage IL-12 production

Macrophages can either facilitate tumor progression or tumor rejection, depending on how they have been activated. In most tumor-bearing individuals, tumor-associated macrophages (TAMS or M2 macrophages) promote tumor progression by their elevated production of IL-10 and minimal production of IL-12 and nitric oxide (NO). In vitro activation with IL-4 and IL-13 through the alternative pathway, gives a similar profile. In contrast, macrophages activated via the classical pathway with LPS and IFNγ or macrophages from IL-4Rα-deficient mice (M1 macrophages) promote tumor rejection through their production of high levels of IL-12 and NO, and minimal amounts of IL-10 (21, 22). One of the mechanisms by which MDSC facilitate tumor progression is through their cross-talk with macrophages, resulting in a decrease in macrophage production of IL-12 (18). To determine if inflammation is involved in the MDSC-mediated down-regulation of IL-12, IL-12 production was measured in cultures of peritoneal macrophages from BALB/c mice activated with LPS and IFNγ in the presence of MDSC. MDSC were obtained from the blood of BALB/c mice with 4T1 mammary carcinoma, or from BALB/c mice with 4T1/IL-1β tumor cells. We have previously demonstrated that 4T1/IL-1β tumors produce a heightened pro-inflammatory tumor microenvironment (5). MDSC from 4T1-bearing mice decreased macrophage production of IL-12 by 33%, while MDSC from mice with 4T1/IL-1β tumors decreased macrophage IL-12 production by 84% **(Figure 1).** The IL-12 is produced by the macrophages, and not by the MDSC, since MDSC in the absence of macrophages, either with or without LPS and IFNγ, do not produce IL-12. Experiments using MDSC from IL-1R antagonist-deficient (IL-1Ra$^{-/-}$) mice, which have heightened inflammation due to their inability to down-regulate IL-1β responses, showed similar down-regulation of IL-12 (data not shown). Therefore, MDSC induced in the presence of heightened inflammation are more potent

inhibitors of macrophage IL-12 production, indicating that inflammation through its action on MDSC, limits production of this type 1 cytokine in tumor-bearing individuals.

Inflammation-induced MDSC produce elevated levels of IL-10

The ability of MDSC to down-regulate macrophage production of IL-12 is dependent on MDSC synthesis of IL-10 (18). Therefore, inflammation may decrease macrophage IL-12 production by increasing MDSC production of the type 2 cytokine IL-10. This hypothesis was tested by measuring IL-10 in the supernatants of peritoneal macrophages co-cultured with MDSC in the presence of LPS and/or IFNγ. MDSC were obtained from the blood of BALB/c mice with 4T1 or 4T1/IL-1β tumors **(Figure 2A),** or from the blood of BALB/c or IL-1Ra$^{-/-}$ mice with 4T1 mammary tumors **(Figure 2B)**. As previously observed (18), co-cultures of MDSC from 4T1 tumor-bearing mice with classically activated macrophages produced significant amounts of IL-10. However, MDSC from tumor-bearing mice with heightened inflammation (i.e. BALB/c mice with 4T1/IL-1β tumors, or IL-1Ra$^{-/-}$ mice with 4T1 tumors), produced significantly more IL-10. Additionally, these MDSC from heightened inflammatory environments and activated with LPS and IFNγ in the absence of macrophages produced significantly more IL-10, than MDSC from less inflammatory environments (i.e. MDSC from BALB/c mice with 4T1 tumors). Previous experiments using IL-10-deficient macrophages and MDSC demonstrated that the IL-10 in macrophage-MDSC co-cultures is produced by the MDSC, and not by the macrophages (18). This conclusion was supported by the current findings because LPS and IFNγ-activated macrophages did not express significant amounts of IL-10. These results demonstrate that inflammation exacerbates MDSC production of IL-10, and enhances the ability of macrophages to up-regulate MDSC production of IL-10, thereby further skewing immunity towards a type 2 tumor-promoting phenotype.

Inflammation up-regulates CD14 expression on MDSC

The results of Figures 1 and 2 demonstrate that MDSC are activated by LPS and IFNγ. LPS activation occurs through the binding of LPS to the LPS binding protein (LBP) which transfers LPS to CD14 (reviewed in (2)). CD14 then associates with the TLR4 and other co-receptors to mediate LPS signaling (23-25). Signaling through this pathway activates NFκB, and leads to the induction of several inflammatory mediators, including IL-1, IL-6, COX-2, and iNOS (reviewed in (26)). The LPS/TLR4 signaling pathway is well documented in macrophages and dendritic cells; however, it has not been previously described in MDSC. To determine whether inflammation up-regulates IL-10 in MDSC through this pathway, $Gr1^+CD11b^+$ MDSC **(Figure 3A)** from the blood of 4T1 and 4T1/IL-1β BALB/c tumor-bearing mice, and MDSC from 4T1 $TLR4^{-/-}$ tumor-bearing mice were cultured in the presence or absence of LPS and IFNγ, and the expression of TLR4 and CD14 was determined by flow cytometry. TLR4 expression is equivalent on MDSC induced by 4T1 and 4T1/IL-1β tumor cells and does not change with LPS and IFNγ treatment (**Figure 3B**). In contrast, CD14 expression is elevated by LPS and IFNγ treatment, and is highest on $Gr1^+CD11b^+$ MDSC induced by 4T1/IL-1β tumor cells (**Figure 3C**). MDSC from TLR4-deficient mice, which are unresponsive to LPS (27), do not significantly up-regulate CD14. Therefore, inflammation increases MDSC expression of CD14 through a TLR4-dependent mechanism

Since CD11b is also known to interact with TLR4 (28) and to enhance responsiveness to LPS (29), MDSC induced by 4T1 or 4T1/IL-1β tumor cells in wild type BALB/c or $TLR4^{-/-}$ mice were also analyzed for expression of CD11b (**Figure 3D**). CD11b levels are elevated by LPS and IFNγ treatment; however, there is no difference in expression of CD11b based on the type of tumor or host,

suggesting that heightened inflammation does not mediate its effects on MDSC through differential regulation of CD11b expression.

Since LPS activation of immature myeloid cells can induce their differentiation to DC or macrophages, LPS and IFNγ-treated MDSC from BALB/c mice with 4T1 tumors were tested for their expression of the macrophage and DC markers F4/80, CD11c, and DEC205. The cells were also tested for the co-stimulatory molecules CD80 and CD86 which are elevated on mature DC and activated macrophages, as well as on MDSC (5, 7, 30) (**Figure 3E**). F4/80, CD11c, and DEC205 levels on MDSC from 4T1 tumor-bearing mice remained unchanged following a 24 hr stimulation with LPS and IFNγ, whereas CD80 and CD86 levels increased. Similar results were seen for MDSC from BALB/c mice with 4T1/IL-1β tumors (data not shown). These results demonstrate that LPS plus IFNγ treatment of MDSC does not induce MDSC differentiation to macrophages or DC. Therefore, LPS plus IFNγ increase CD14 expression on MDSC, suggesting that inflammation may mediate its effects on MDSC through the CD14/TLR4 pathway.

Inflammation activates NFκB in MDSC

Signaling through the TLR4 pathway leads to the activation of NFκB which in turn activates genes which contribute to inflammation. NFκB is sequestered in the cytoplasm in association with the inhibitory subunit IkBα, but is released for translocation to the nucleus by phosphorylation and subsequent proteosomal degradation of IkBα (reviewed in (31)). If LPS and inflammation are increasing MDSC activity by signaling through the TLR4 pathway, then MDSC activated in heightened inflammatory settings may have elevated levels of activated NFκB in the nucleus. To test this hypothesis, the levels of phosphorylated IkBα (pIkBα) and total IkBα protein were measured by western blot. MDSC from BALB/c mice with either 4T1 or 4T1/IL-1β tumors were untreated or

treated for 24 hours with LPS and IFNγ, and cell lysates were immunoblotted with antibodies to pIkBα or IkBα **(Figure 4)**. HeLa cells stimulated with TNFα were used as a positive control for expression of total IkBα protein and pIkBα. Untreated MDSC from BALB/c mice with 4T1 or 4T1/IL-1β tumors expressed similar levels of total IkBα protein; however, MDSC from mice with 4T1/IL-1β tumors expressed 3 times more pIkBα than MDSC from mice with 4T1 tumors (ratio of IkBα to pIkBα: 0.1 and 0.299 for 4T1 and 4T1/IL-1β MDSC, respectively). Similarly, MDSC from mice with 4T1/IL-1β tumors stimulated with LPS and IFNγ expressed elevated levels of pIkBα compared to MDSC from mice with 4T1 tumors (ratio 0.37 and 0.2, respectively). MDSC induced by heightened inflammation also have elevated levels of pIkBα, and these levels are enhanced by treatment with LPS and IFNγ. Therefore, inflammation activates NFκB in MDSC, and is likely to result in the up-regulation of additional pro-inflammatory mediators.

Inflammation-induced increase of IL-10 in MDSC and decrease of IL-12 in macrophages are TLR4-dependent

If inflammation is increasing MDSC production of IL-10 through a CD14/TLR4/NFκB signaling pathway, then MDSC from TLR4-deficient tumor-bearing mice with heightened inflammation should not have elevated levels of IL-10. Likewise, if MDSC reduce macrophage production of IL-12 through a CD14/TLR4 signaling pathway, then MDSC from TLR4-deficient tumor-bearing mice should not decrease macrophage production of IL-12. This hypothesis was tested by comparing IL-10 and IL-12 levels in LPS and IFNγ-treated co-cultures of BALB/c macrophages with MDSC from BALB/c mice with 4T1 or 4T1/IL-1β tumors, or with MDSC from $TLR4^{-/-}$ mice with 4T1 tumors. As previously observed in Figure 2, LPS-activated, inflammation-induced MDSC produce elevated levels of IL-10 both in the presence and absence of macrophages (4T1/IL-1β vs. 4T1

MDSC). In contrast, MDSC from TLR4$^{-/-}$ mice produce no IL-10, regardless of the presence of LPS and IFNγ, or inflammation (**Figure 5A**). Similarly, inflammation-induced MDSC from BALB/c mice are very potent inhibitors of macrophage production of IL-12 (4T1/IL-1β vs. 4T1 MDSC), as previously seen in Figure 1, whereas MDSC from TLR4$^{-/-}$ mice are much less effective in reducing macrophage production of IL-12 (**Figure 5B**). Therefore, inflammation enhances the ability of MDSC to secrete IL-10 and to down-regulate macrophage production of IL-12 through an LPS/TLR4-dependent pathway in the MDSC.

Macrophage induction of IL-10 by MDSC is TLR4 dependent

To determine if the effects of macrophages on MDSC are also regulated through the LPS/TLR4/NFκB pathway, IL-10 and IL-12 levels were measured in co-cultures of TLR4$^{-/-}$ macrophages and MDSC from BALB/c mice with 4T1 tumors (**Figure 5C**). As expected, LPS-treated TLR4$^{-/-}$ macrophages do not produce significant levels of IL-12 because they are unable to respond to LPS. In contrast to earlier findings with BALB/c macrophages (**Figure 2**), TLR4$^{-/-}$ macrophages do not increase MDSC production of IL-10. Therefore, the cross-talk between MDSC and macrophages that promotes a tumor-promoting cytokine phenotype of high IL-10 and low IL-12 is regulated by the TLR4 signaling pathway in both MDSC and macrophages.

DISCUSSION

The TLR4 and NFκB pathway has been well documented in mature myeloid cells, such as macrophages, but has not been previously identified in myeloid-derived suppressor cells. We now report that inflammation activates MDSC through the TLR4-CD14-NFκB pathway, and that activation of this pathway in MDSC enhances the cross-talk between MDSC and macrophages in which macrophages up-regulate MDSC production of IL-10 and MDSC down-regulate macrophage production of IL-12. Since increases in IL-10 and decreases in IL-12 direct tumor immunity away from a tumor-rejecting type 1 response and towards a tumor-promoting type 2 response, these findings identify another mechanism by which inflammation, through NFκB, promotes tumor growth.

NFκB is widely recognized as a key regulator of tumor progression; however, whether it promotes or delays tumor growth depends on which cell type it is expressed in, and when it is expressed. NFκB activation in macrophages results in the production of pro-inflammatory mediators that produce a type 1 anti-tumor immune response (26, 32), and contribute to immune surveillance and tumor rejection (33). However, increasing tumor burden inhibits the activation of NFκB in tumor-associated macrophages (TAMS), thereby polarizing TAMS towards a tumor-promoting M2 phenotype (34, 35). In contrast, inactivation of NFκB in tumor cells blocks tumor development in models of liver, colon, and mammary cancer, demonstrating that tumor cell expression of NFκB enhances tumor progression (36-38). Inactivation of NFκB in tumor cells may delay tumor promotion and progression by enhancing apoptosis (37), and/or by shifting the balance from the production of tumor-promoting pro-inflammatory TNFα towards TRAIL-mediated apoptosis (38). Activation of NFκB in MDSC also contributes to tumor progression; however, it does so by stimulating immune suppression that blocks tumor immunity. It is intriguing that although macrophages and MDSC share a common hemopoietic lineage, the two populations respond very differently to LPS and IFNγ. As

demonstrated in this report, LPS and IFNγ treatment of MDSC promotes a type 2 response with decreases in IL-12 and increases in IL-10. As a result, tumor growth is enhanced through the induction of immune suppression. In contrast, treatment of macrophages and DC with LPS and IFNγ up-regulates MHC II and co-stimulatory molecule expression, and increases macrophage production of IL-12 (31), resulting in a type 1 response (2) that favors anti-tumor immunity and promotes tumor rejection. Although LPS is a differentiation agent for macrophages (2), it is unlikely that the different responses of macrophages and MDSC are due to the lack of plasticity of MDSC, since Gabrilovich and colleagues have shown that MDSC differentiate to mature DC following treatment with all-trans-retinoic acid (10). The MDSC response to LPS is also unlikely to be due to LPS-triggered de-sensitization, a phenomenon which has been reported in macrophages (39). Macrophage tolerance to LPS is accompanied by increases in CD14 expression (40) and IL-10 production (39), similar to what occurs for MDSC. However, NFκB is down-regulated in macrophages that are tolerant to LPS, while NFκB activation is increased in LPS-treated MDSC. Therefore, the differential responses of macrophages and MDSC to LPS are not due to different maturation states or developmental plasticity, or to LPS de-sensitization.

Although LPS is sufficient for signaling through TLR4, the combined treatment of MDSC with LPS and IFNγ gives the maximal response. Increases in CD14 expression enhance responsiveness to LPS by reducing the concentration of LPS required for TLR4 signaling (39). Up-regulation of CD14 also promotes survival by protecting against apoptosis (41). LPS treatment increases CD14 levels on MDSC; however, it also down-regulates TLR4 expression (42). Since IFNγ increases expression of TLR4 and its associated adaptor protein, MD-2 (42), it may prevent the LPS-mediated down-regulation of TLR4, and thereby maintain NFκB activation through TLR4. Previous studies showing that IFNγ knockout mice produce MDSC that are less suppressive than MDSC from wild type mice

(7), support the concept that IFNγ boosts MDSC activity, but is not essential for the production of MDSC.

The experiments described in this report used LPS and IFNγ to activate inflammation-induced MDSC in vitro, raising the question of which molecules activate MDSC through the TLR4-NFκB pathway in vivo. Chronic inflammatory conditions are frequently triggered and/or maintained by infectious pathogens (43), which have the potential to produce molecules such as LPS. However, the etiology of many inflammatory conditions is unknown. In these cases, stimulation of TLR4 on MDSC may be due to endogenous ligands released by injured or necrotic tissues, such as High-mobility group box 1 protein (HMGB1), a pro-inflammatory cytokine released by necrotic cells (44), heat shock proteins 60, 70, or 90, heparin sulfate, or soluble hyaluronan (45). These molecules have been implicated as TLR4 ligands that activate the NFκB pathway, so it is not unreasonable that they would also activate MDSC through TLR4. Interestingly, paclitaxel is also a TLR4 ligand, raising the possibility that certain cancer chemotherapeutic drugs may actually promote tumor progression by activating MDSC (46).

Tumor progression is a balance between many factors, only some of which are regulated by inflammation and NFκB. However, the finding reported here that inflammation-induced activation of NFκB in MDSC promotes immune suppression, provides additional support for developing therapies that target NFκB as a strategy to down-regulate multiple factors that favor tumor growth.

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FOOTNOTES:

Abbreviations used in this study:

4T1/IL-1β,	4T1 cells transduced with ssIL-1β
IkBα,	inhibitor of nuclear factor kappa-B, alpha
iNOS,	inducible nitric oxide synthase
IL-1ra,	interleukin 1 receptor antagonist
MDSC,	myeloid-derived suppressor cell(s)
TAMs,	tumor-associated macrophages
TLR4,	toll-like receptor 4

FIGURE CAPTIONS

Figure 1. Inflammation enhances the ability of MDSC to down-regulate macrophage production of IL-12. BALB/c mice were inoculated with 7000 4T1 or 4T1/IL-1β mammary carcinoma cells and bled when their primary tumors were 8-10 mm in diameter. The resulting nucleated cells, labeled as 4T1 or IL-1β, respectively, in the MDSC column, were >88% $Gr1^+CD11b^+$ and were co-cultured with peritoneal macrophages from tumor-free BALB/c mice for 24 hrs. in the presence or absence of LPS and/or IFNγ. Supernatants were tested for IL-12 by ELISA. Data are from one of 2-5 independent experiments.

Figure 2. Inflammation increases IL-10 production by LPS-stimulated MDSC. BALB/c and $IL\text{-}1Ra^{-/-}$ mice were inoculated with 7000 4T1 or 4T1/IL-1β mammary carcinoma cells and bled when their primary tumors were 8-10 mm in diameter. The resulting nucleated cells were >85% $Gr1^+CD11b^+$ MDSC and were cultured with peritoneal macrophages from tumor-free BALB/c mice for 24 hrs. in the presence or absence of LPS and/or IFNγ. Supernatants were tested for IL-10 by ELISA. (A) MDSC from BALB/c mice with 4T1 or 4T1/IL-1β tumors co-cultured with BALB/c macrophages, labeled as 4T1 and IL-1β, respectively, in the MDSC column. (B) MDSC from BALB/c or $IL\text{-}1Ra^{-/-}$ mice with 4T1 tumors, labeled as 4T1 and $IL\text{-}1Ra^{-/-}$, respectively, in the MDSC column. Data are from one of 2-5 independent experiments.

Figure 3. LPS up-regulates CD14 expression by MDSC. Mice were inoculated with 7000 4T1 or 4T1/IL-1β mammary carcinoma cells and bled when their primary tumors were 8-10 mm in diameter. The resulting nucleated cells were cultured with or without LPS and/or IFNγ, fluorescently labeled for Gr1, CD11b, and other cell surface markers and analyzed by flow cytometry. (A) Gated $Gr1^+CD11b^+$

MDSC analyzed in panels B-D. (B) Nucleated cells were incubated for 24 hrs without LPS and IFNγ, with LPS and IFNγ, or with IFNγ alone, stained for Gr1, DC11b and TLR4, and the gated $Gr1^+CD11b^+$ MDSC analyzed for TLR4 expression. (C) Nucleated cells were incubated for 4 or 24 hrs without LPS and IFNγ, with LPS and IFNγ, or with IFNγ alone, stained for Gr1, CD11b, and CD14, and the gated $Gr1^+CD11b^+$ MDSC analyzed for CD14 expression. Isotype control and CD14 staining were equivalent for MDSC incubated in the absence of LPS and IFNγ so only one trace is shown. In the $TLR4^{-/-}$ MDSC panels, isotype (dotted line) and CD14 (black line) without LPS or IFNγ are shown as separate traces, and CD14 with LPS and IFNγ (shaded area) is shown. (C) Nucleated cells were incubated for 1 or 24 hrs without LPS and IFNγ, with LPS and IFNγ, or with IFNγ alone, stained for Gr1 and CD11b, and the Gr1-gated cells analyzed for CD11b expression. Isotype control staining was equivalent for cells incubated with or without LPS and IFNγ, so only one trace is shown. (D) Nucleated cells from 4T1 tumor-bearing BALB/c mice were incubated for 24 hrs with or without LPS and IFNγ, stained for Gr1, CD11b, and the indicated surface markers, and the gated $Gr1^+CD11b^+$ cells analyzed. Data are from one of 3-5 independent experiments.

Figure 4. Inflammation increases the amount of phosphorylated IκBα in $Gr1^+CD11b^+$ MDSC. BALB/c mice were inoculated with 7000 4T1 or 4T1/IL-1β mammary carcinoma cells and bled when their primary tumors were 8-10 mm in diameter. The resulting nucleated cells were 90% $Gr1^+CD11b^+$ MDSC and were incubated for 24 hrs with or without LPS and IFNγ, lysed, and the lysates, analyzed by Western blotting with antibodies to phospho-IκBα and IκBα. HeLa cells activated with IFNα were run as a positive control for phospho- IκBα protein. Ratios of IκBα to phospho-IκBα for non-activated MDSC from 4T1 and 4T1/IL-1β–bearing mice are 0.1 and 0.299, respectively. Ratios of

IκBα to pIκBα for LPS and IFNγ activated MDSC from 4T1 and 4T1/IL-1β–bearing mice are 0.2 and 0.37, respectively. Data are from one of 3-5 independent experiments.

Figure 5. Cross-talk between MDSC and macrophages requires TLR4. Mice were inoculated with 7000 4T1 or 4T1/IL-1β mammary carcinoma cells and bled when their primary tumors were 8-10 mm in diameter. The resulting cells were >85% $Gr1^+CD11b^+$ and were co-cultured for 24 hrs. with peritoneal macrophages from BALB/c or $TLR4^{-/-}$ mice in the presence or absence of LPS and/or IFNγ. (A) $Gr1^+CD11b^+$ MDSC from BALB/c mice with 4T1 or 4T1/IL-1β tumors and MDSC from $TLR4^{-/-}$ mice with 4T1 tumors were co-cultured with BALB/c macrophages and culture supernatants were assayed for IL-10 by ELISA. (B) $Gr1^+CD11b^+$ MDSC from BALB/c mice with 4T1 or 4T1-IL-1β tumors and MDSC from $TLR4^{-/-}$ mice with 4T1 tumors were co-cultured with BALB/c macrophages and culture supernatants were assayed for IL-12 by ELISA. (C) $Gr1^+CD11b^+$ MDSC from BALB/c mice with 4T1 tumors were co-cultured with $TLR4^{-/-}$ macrophages and supernatants were assayed for IL-10 and IL-12 by ELISA. Data are one of 2-3 independent experiments.

Figure 1: Inflammation enhances the ability of MDSC to down-regulate macrophage production of IL-12.

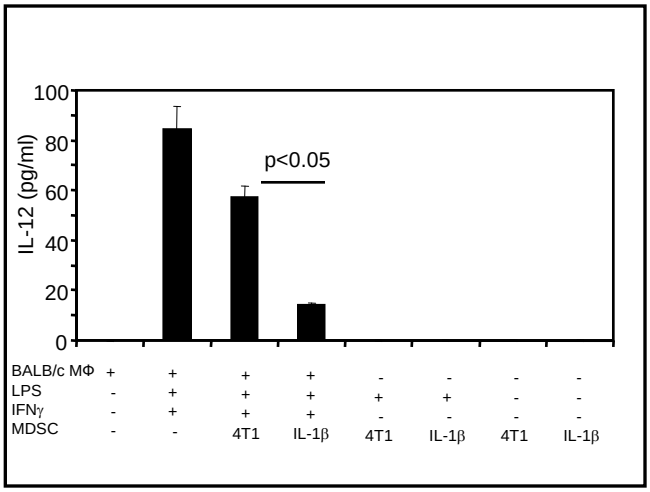

Figure 2: Inflammation increases IL-10 production by LPS-stimulated MDSC.

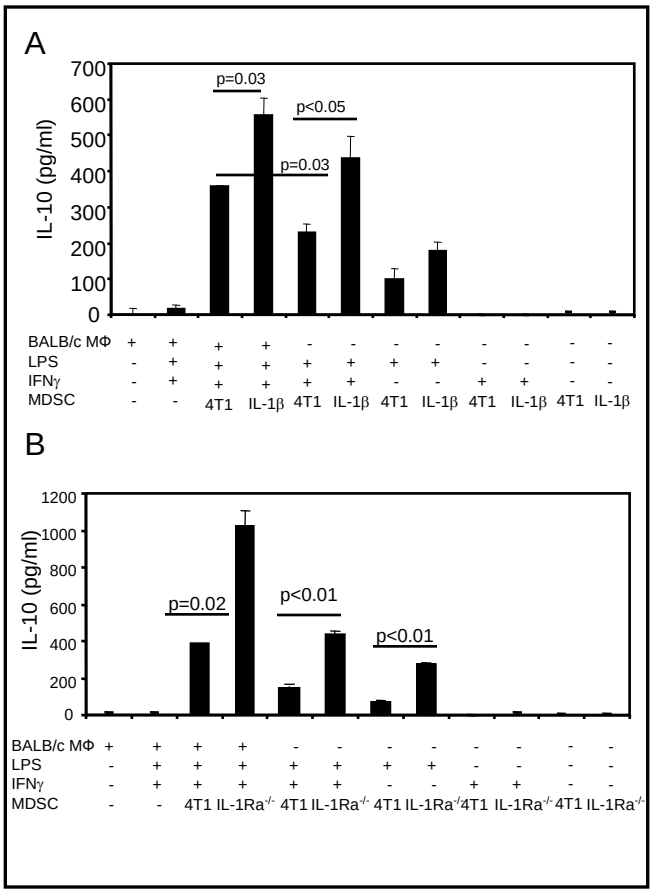

Figure 3: LPS up-regulates CD14 expression by MDSC.

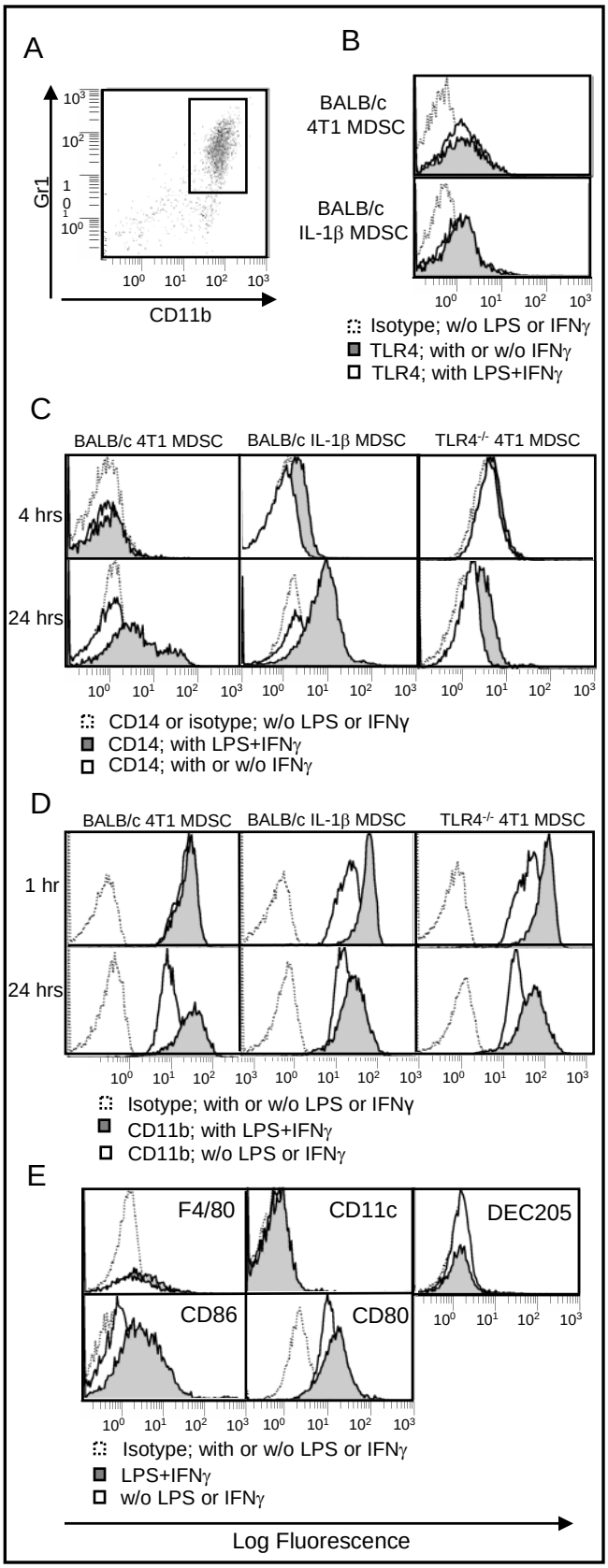

Figure 4: Inflammation increases the amount of phosphorylated IκBα in $Gr1^{+}CD11b^{+}$ MDSC.

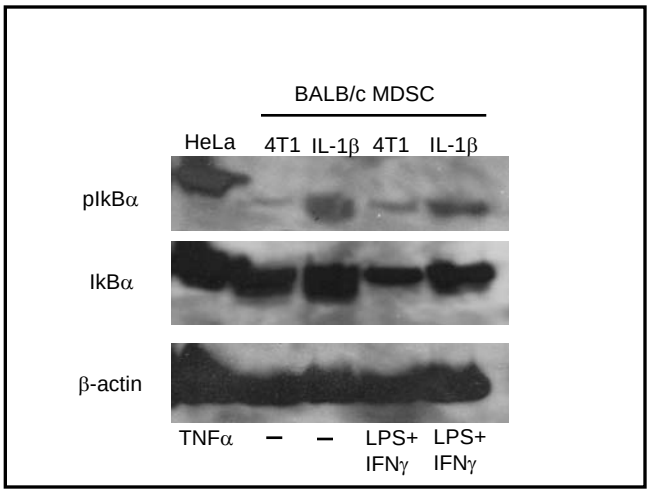

Figure 5: Cross-talk between MDSC and macrophages requires TLR4.